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LEUKOCYTE CAPILLARY AGGLUTINATION:
APPLICATION IN TRANSPLANTATION IMMUNOLOGY.

BY



LESLIE ANNE OLSON

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
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ABSTRACT

The technique of leukocyte capillary agglutination, as originally described by Thompson, was compared to lymphocytotoxicity (Terasaki's method) and leucoagglutination (Rose Payne's technique) with respect to sensitivity, specificity, and relative serum changes with the passage of time. Capillary agglutination detects antibodies which prevent mechanically aggregated leukocytes in capillary tubes from disaggregating with the force of gravity.

In studying the anamnestic response in postmenopausal multiparous women, it was shown that sensitization to HL-A antigens may become latent, with a prompt humoral response being evoked by subsequent exposure. The implication is that the same could happen in the case of renal allograft recipients under those circumstances where prior HL-A sensitization would not be detected by the lymphocytotoxic pre-transplant crossmatch.

There was no consistent relation between the three methods with respect to sensitivity or relative serum changes with the passage of time. There was a significant difference, in compared sera, between the specificity of each method. Only 50% of antibodies in the sera of parous women were detectable by lymphocytotoxicity. Capillary agglutination (CAT) correlated with both a lack of rejection in renal transplant recipients and the "enhancing" antibody as defined by inhibition of the unidirectional mixed lymphocyte culture (UMLC). CAT also differentiated between HL-A identical siblings in a manner correlating with MLC reactivity. Both capillary agglutinating and lymphocytotoxic activity was found in the IgG fraction of serum. Capillary agglutination could be very useful in transplantation immunology studies, if the

exact nature of the antibody detected could be defined and if the method was further automated.

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CHAPTER I

INTRODUCTION

The renal transplant results before 1966 show a progressive improvement in patient survival and graft function (Lazarus and Hampers, 1972). Some of the reasons for this are the use of the pre-transplant donor lymphocyte-recipient serum crossmatch, the time of warm ischemia, the use of immunosuppressive drugs, and management of the patients. However, since 1966 there has been a plateau in the survival curve, shown in Table I of Appendix A (Advisory Committee to the Renal Transplant Registry, 1972). This plateau is despite histocompatibility studies which led to better matched kidney allografts, the use of anti-lymphocyte globulin, thoracic duct lymphocyte depletion, and the surgical experience (Lazarus and Hampers, 1972).

There are several possible reasons for this lack of continued improvement. First, all of the HL-A antigens (Histocompatibility Locus-A) are probably not yet detectable and thus cannot be matched. Second, there may be other histocompatibility systems besides HL-A of importance in transplantation. Since skin grafts are rejected between HL-A identical siblings, it has been postulated that there may be weaker loci present (as in the mouse) (Amos et al, 1969). A third reason may be that humoral pre-sensitization to HL-A antigens may be present in a latent state, affecting transplant success. Fourthly, previously established cellular immunity to HL-A antigens may be present in a recipient and, as yet, this form of pre-sensitization cannot be detected. A fifth reason may be the presence of organ-specific antibodies not detected in

the lymphocyte-detection systems. A sixth reason could be the failure to recognize that some types of antibodies may be "good" or "enhancing" in the allograft situation.

One approach in the attempt to improve kidney transplant success is to explore the value of new techniques in detecting, for example, antigens of other histocompatibility loci, latent sensitization to HL-A, or "enhancing" antibodies. One such technique is the leukocyte capillary agglutination test (CAT) of Thompson and Severson. They introduced it first in mice, in 1966 (Severson and Thompson, 1966); then in humans in 1968 (Thompson et al, 1968). It was developed as a more sensitive technique to detect leucoagglutinins or, more exactly, antibodies that prevent mechanically aggregated leukocytes in capillary tubes from disaggregating with the force of gravity (Severson and Thompson, 1966). Besides being more sensitive and reproducible than leucoagglutination, the technique is quantitative and can be adapted to automation and computer analysis (Thompson et al, 1968). Furthermore, in 1970 they suggested that some cytotoxically monospecific sera are heterogeneous with respect to CAT antibodies (Thompson et al, 1970). In a few cases (Thompson et al, 1971), these additional reactions are detecting antigens which are not related to the HL-A locus, the neutrophil antigens NAI, NBI and NCI of Lalezari (1966a, 1966b, 1970), or the 5a and 5b antigens of van Rood (1964). Thus, there were indications that this technique could be of use in detecting non-cytotoxic antibodies (non-HL-A? latent? "enhancing"?) and further characterization of the CAT technique was undertaken for this project.

Its value as a tissue typing method had to be determined in comparison with lymphocytotoxicity and leucoagglutination, the two

commonly used methods. It was deemed important to know whether CAT antibodies were equivalent to cytotoxic antibodies with respect to specificity, threshold of detection (sensitivity), the class of antibody involved (IgG or IgM), and correlation with the clinical progress of kidney transplant recipients. An important question which evolved during the research was whether these antibodies correlated with the "blocking" or "enhancing" antibody as defined by inhibition of the mixed lymphocyte culture reaction (MLC) and the modification by which mitomycin C treatment or X-irradiation is used to prevent blastogenesis in one of the cell types, thus converting the MLC into a unidirectional MLC response (or UMLC) (Ceppellini et al, 1971; Hattler et al, 1971).

For purposes of comparison, the three humoral tests were studied in various situations:

1. In postmenopausal multiparous women, who had no residual lymphocytotoxic antibodies, we examined the response to the blood transfusions required at surgery, to see if the antibody response was anamnestic, indicative of latent HL-A sensitization.
2. The sera of dialysis patients and kidney transplant recipients were tested each month for the presence of antibody, using a panel of lymphocytes from normal persons in the laboratory (the monthly "screening").
3. Sera obtained at delivery from parous women were screened for antibody.

In addition, the CAT antibody was characterized by the following:

1. The sensitivity, and the rate of appearance and decay of the CAT antibody were studied in comparison with lymphocytotoxicity (CYT) and leukoagglutination (AGG).

2. The HL-A specificity of CAT was examined by analyzing serum reactions by all three tests (CYT, CAT and AGG) and by studying the inheritance pattern in a large Eskimo family.

3. Sera and IgG from multiparous women and renal patients respectively were used to compare CYT and CAT antibody with UMLC inhibition and antibody dependent cell immunity (ABCIL).

4. Serum fractions IgG and IgM were tested for capillary agglutinating and cytotoxic activity.

CHAPTER II

LITERARY REVIEW

A. Histocompatibility Antigens

Medawar, in 1946, was the first to demonstrate that there are transplantation antigens on leukocytes (Medawar, 1946). This classic study showed allograft sensitivity was induced in rabbits by the intradermal injection of buffy coat. These findings were confirmed for humans by Rapaport et al (1961) and Friedman et al (1961). Although graft rejection is largely cell mediated, and allograft sensitization cannot be transferred by serum (Weiser et al, 1969), leukocyte antibodies develop after graft rejection in mice, as shown by Amos (1953) and Gorer (1956), and after human graft rejection as detected by van Rood et al by the leukoagglutination test (1964), Walford et al (1964) and Terasaki et al (1964a) by lymphocytotoxicity, and Colombani et al using the antiglobulin consumption test (1964).

Leukocyte antigens are present on most cells and tissues of the body, providing additional evidence that they are transplantation, or histocompatibility, antigens. Seigler and Metzgar have studied the embryonic development of these antigens, detecting them on the six-week fetus (1970). In older fetuses, they found leukocyte antigens on each organ, though some antigens were masked or deficient on some organs.

Berach et al studied the distribution of HL-A2 (a leukocyte antigen) in human organs (1970). It was most abundant in spleen, then lung, liver, intestine, kidney, and heart. Leukocyte antigens are on platelets, but not on red blood cells, and are most abundant on leukocytes,

reticuloendothelium, and the epithelium of the skin (Seigler and Metzgar, 1970). Thus leukocyte antigens are present on all nucleated cells, but, in man as distinct from the mouse, they are absent from the red blood cells.

The best evidence for leukocyte antigens being histocompatibility antigens is in skin and renal grafting results. It was mainly Dausset et al (1965b) and Dausset and Rapaport (1966) who showed that compatible skin grafts survive longer, with or without preimmunization. More recently, it appears that the most successful kidney grafts between blood relatives are those most compatible with respect to leukocyte antigens. This is discussed further in section D of this Chapter.

Leukocyte antigens, then, are important as histocompatibility antigens. However the ABO erythrocyte system must also be considered. Rapaport et al preimmunized recipients with ABO incompatible red cells (1968). The incompatible skin grafts were then rejected more rapidly than compatible ones. Even without preimmunization, ABO incompatible skin grafts are quickly rejected (Ceppellini et al, 1969). In kidney transplants, there is usually rapid rejection with ABO incompatibilities (Starzl et al, 1964), and the ABO barrier is not crossed in transplantation.

1. Leukocyte antigens (HL-A)

In 1954, Dausset discovered leucoagglutinins developing after blood transfusions, and found that these antibodies reacted with only some cell types (Dausset, 1958). He identified the first leukocyte iso-antigen, called Mac, by immunizing several recipients (1958). This antigen occurred in about 60% of the French population, and is now known as HL-A2.

The search began for additional leukocyte antigen specificities, using the leukoagglutination test as developed by Dausset (1958), Payne, using defibrinated blood (1957), or van Rood and van Leeuwen (1963) using EDTA as an anticoagulant.

Payne first demonstrated leukoagglutinin formation in the multiparous woman in 1958 (Payne and Rolfs, 1958), and this has been the major source of histocompatibility antisera. Van Rood et al (1959) confirmed the finding and showed the antibodies are directed against the husband's antigens, so they are more likely to be monospecific than those formed after transfusion.

Most early tissue typing antisera were multispecific, but there were some consistent reaction patterns. Van Rood and van Leeuwen first outlined the leukocyte antigen locus (1963). They analysed by computer the reactions of several selected (oligospecific) sera against a large panel of test cells, using chi-square analysis, and found two groups of sera, all the sera in each group reacting in a comparable way. They called the antigens identified by these sera 4a and 4b and each cell was 4a, 4b or 4ab. They postulated two codominant alleles for antigens 4a and 4b; they named the locus the "4" locus.

Various types of computer analysis, such as factor analysis (Terasaki et al, 1965, Mickey et al, 1968) and the posterior probabilities method (Mickey et al, 1967) were thereafter used to select the sera most likely to recognize single antigens. Absorption studies are then used to determine monospecificity (Terasaki and Singal, 1969). The many laboratories working with leukocyte antigens before 1965 were using somewhat different techniques, and detecting antigen specificity by one method only, making it impossible to correlate results between labor-

atories. Each laboratory had (and some still do have) its own terminology, which they arbitrarily changed periodically, creating an increasingly confusing situation.

The techniques of antibody detection were expanded to include the platelet complement fixation method of Shulman (1964) and the lymphocytotoxicity method of Terasaki (1964a), Kissmeyer-Nielsen (1968) and Amos (1965). The latter technique, which readily detects antibodies occurring after pregnancy and multiple transfusions (Terasaki et al, 1964b), was adopted by all research teams and at international histocompatibility workshops, and antigen identification and correlation then progressed rapidly. Whether universal adoption of lymphocytotoxicity was a wise decision is one of the questions to be considered in this thesis; at the time the value of universal adoption of a standardized technique outweighed all other considerations.

At the first of these workshops, in 1965 in Leiden (Balner et al, 1965), Dausset's original "Mac" antigen was found to correlate with Payne's LA2 (1964), van Rood's 8a (1965), Terasaki's group 2 (1965), and Shulman's PI_{Gr}Ly^{BI} (1962). It was given the nomenclature HL-A2 (Nomenclature Committee, 1968).

The diallelic antigens 5a and 5b were described by van Rood in 1964 (van Leeuwen et al, 1964); 5a having a gene frequency of 0.20 and 5b with a frequency of 0.80. He concluded that 5a is not a transplantation antigen and is a poor stimulator of antibody formation during pregnancy (van Rood et al, 1965). Anti-5a sera are rare; however anti-5b sera are fairly common. Lawler and Shatwell (1967) found 5b to be strongly antigenic in pregnancy. Though found on other cells than granulocytes, the 5a and 5b antigens are detectable only by agglutination,

never by lymphocytotoxicity (van Leeuwen et al, 1964). They have little relationship to the other antigens.

The 6 and 7 systems were also identified by van Rood et al in 1965. Their Group 6 was triallelic, with alleles 6a, 6b and 6c. They thought it could be closely linked to Group 4. The antigens of their Group 7 (7a, 7b, 7c = 6c, 7d) fit a four-allele genetic model. It was then seen that both of these systems may be related to Group 4. 7b "included" 4a; 7a, 7d, 6b and 7c were "included" in 4b; 6a was thought to be associated with 4a and 4b. Within the last year, further analysis has led to the concept of supertypes and subtypes within the "4" series, including all the Group 6 and 7 allelic specificities as well (D'Amaro et al, 1972). As an example, supertype 4a includes the antigens HL-A5, 12, 13, 17, W18, 27 and SL*. This is seen in Appendix A, Figure 1. Another antigen, 9a, was also identified (van Rood et al, 1965). It was later found to be granulocyte-specific.

Payne et al of Los Angeles named the LA1 and LA2 antigens in 1964, as two groups of negatively associating sera by chi-square analysis. LA1 became known as HL-A1 (Nomenclature Committee, 1968) and LA2 was identical to HL-A2 (Balner et al, 1965). Bodmer and Payne also characterized the 4c and 4d antigens (1965). 4c is almost completely "included" in van Rood's 4a, and 4d is similarly "included" in van Rood's 4b.

In 1964, Payne et al suggested independence of the LA and 4 systems (Payne et al, 1964). The LA system consisted then of LA1 and LA2 and the 4 system was 4a, b, c, and d. In 1965 they described the LA3 antigen (Bodmer et al, 1966), which is probably controlled by an allele LA3 codominant with LA1 and LA2. It became known as HL-A3 by international workshop agreement (Nomenclature Committee, 1968). LA4 was

identified in 1967 (Curtoni et al, 1968) and became HL-A9 (Terasaki, 1970).

Dausset et al proposed the existence of one complex histocompatibility locus, Hu-1, in 1965 (1965a). They could classify 49 of 50 sera into two groups of positive correlations (subloci). Within each group are subgroups of sera with positive correlations. They proposed a limited number of antigens of each sublocus, with several subloci possible. Since that time, the system has been named HL-A, or Histocompatibility Locus-A (Amos, 1968; Curtoni et al, 1968). Various family studies have shown it to consist of one locus with two subloci of mutually exclusive antigenic specificities (Singal et al, 1968; Svejgaard et al, 1970; Dausset et al, 1969). These are called subloci 1 and 2, or series "LA" and "Four". Every cell has two alleles from each sublocus, one allele of each on each chromosome. The two alleles on one chromosome, one from each sublocus, constitute an allelic haplotype, inherited from one parent. This is borne out in family studies, where every person may have two antigens from each sublocus, in which event only one antigen from each sublocus comes from each parent (Svejgaard et al, 1970). This inheritance pattern obeys the restrictions of the rules of genetics for a single locus on an autosomal pair of chromosomes.

Among very many families studied for HL-A inheritance, several instances of cross-over have been reported (Kissmeyer-Nielson et al, 1969; Seignalet, 1971). This occurs with a frequency of 0.5 to 1.0% (Svejgaard et al, 1971) and supports the two sublocus idea for HL-A. Some investigators have postulated a third sublocus at different times (Walford et al, 1969; Sandberg et al, 1970), but most now believe that HL-A has only two subloci.

With the successive international computerised workshop analyses of 1965, 1967, 1970, and 1972, the antigens of the two HL-A subloci have been defined with increasing specificity. Many of these antigens bear the International Nomenclature (Nomenclature Committee, 1968), though some have "Workshop numbers" (W). The antigens now identified on each sublocus are as follows:

Sublocus 1: HL-A 1, 2, 3, 9, 10, 11, 28, W29, 31, 33

Sublocus 2: HL-A 5, 7, 8, 12, 13, 14, 17, 27, W15, 16, 18, 21, 22, Te50, Te60.

This is shown in more detail in Table 2 of Appendix A, with previous designations listed for each antigen. It is now believed that the 4a and 4b antigens (Dausset's series 3 and 7) are supertype specificities of the second sublocus, as referred to above. This hypothesis would explain the many phenotypic expressions and the significant statistical association between them. Van Rood states that 4a and 4b can be recognized by monospecific sera, and that they are still useful in determining the degree of identity between a donor and recipient (van Rood et al, 1970).

One system which does not fit into the presently accepted idea of the HL-A locus is the 5a and 5b antigen system, detectable only by agglutination. It is believed to be on a separate locus (van Rood et al, 1968).

Recently, the observation of cross-reactivity in the HL-A system has complicated the definition of the antigens. Some antibodies cross-react with antigens which, with other antibodies, appear to be serologically unrelated. Two pathways are possible for this (Hirschfield, 1968; Kissmeyer-Nielsen et al, 1970): 1) Complex antigens exhibit various but related specificities, each inducing specific simple antibodies; and/or

2) certain simple antigens induce complex and cross-reactive antibodies. Either may explain how pure incompatibilities may yield antibodies that react with antigens not detectable in the donor (Thorsby et al, 1970).

Thorsby et al found several sera containing cross-reacting HL-A antibodies (1970). Some of these sera were produced by planned immunization in which the antibodies cross-react with antigens not possessed by the immunizing donor. Others were formed during pregnancy and cross-react with antigens not possessed by the husband. They feel the most likely explanation is that antibodies are formed to antigenic structures common to, or very similar to, two or more different HL-A antigens (pathway 1). Several groups of HL-A antigens, within which there are often cross-reactions, are HL-A5 and Te50, HL-A2 and HL-A28, HL-A3 and HL-A11, and HL-A7, HL-A27 and HL-A17.

Colombani et al also stress the importance of cross-reactions in defining new HL-A antigens (1970). They describe the Da-6 Cross-reacting Group (CREG) consisting of HL-A5, Da19, 20, 24 and W15 (Da23). All these antigens are completely included in Te50 and W18 (Da6), and Da24 and W15 are included in Da19. In addition to the CREGs described by Thorsby et al (1970), they mention others: HL-A1 and HL-A3; HL-A7 and W22.

Figure 2 of Appendix A shows the presently recognized cross-reactions within each sublocus, as compiled by Terasaki (Sengar, personal communication). There are some complete inclusions and some partial ones. There are no cross-reactions between the subloci.

Batchelor and Sanderson consider most of the HL-A antigens to be complex antigens consisting of several subunits (1970). This could explain both complete and partial serum inclusions, where the range of positive reactions of one serum falls principally within the second

serum's range of positive reactions. By their theory, a cell could have an antibody combining site which has part of two different antigen sub-unit sequences, so that each antibody can combine. Their theory of shifting reference points is also relevant, stating that the antigen reference points selected by the HL-A serologists are arbitrary. Secondary reference sera are then selected on the basis of their relationship to these. A shifting of the primary reference points would yield a different but equally valid set of antisera.

2. Non-HL-A Tissue Antigens of Limited Distribution

Platelet-specific histocompatibility antigens have also been detected, the most commonly used method being complement fixation (C'F). Platelet agglutinins were first observed by Harrington et al and Stefanini et al in 1953. Moulinier described the first platelet antigen in 1957, naming it Duzo (cited by Shulman et al, 1962). It reacted with 22% of 82 platelet suspensions.

Shulman et al have shown that neonatal thrombocytopenic purpura is caused by maternal antibodies against fetal platelet antigens (1962). In these cases, erythrocyte and leukocyte counts are normal. Platelet antigen PI^{AI} , identified in 1959, occurs in 98% of the general population (Shulman et al, 1962). Platelets homozygous for PI^{AI} are doubly reactive with anti- PI^{AI} as compared to heterozygous platelets.

Van Loghem reported a platelet antigen, Zw, in 1959 (cited by Shulman et al, 1964). This was since recognized to be the same as PI^{AI} . In 1963, van der Weerd found a platelet antigen allelic to Zw - the pair being named Zw^a and Zw^b (cited by Shulman et al, 1964).

The second antigen identified by Shulman was PI^{BI} (1962), originally called PI^{BI} before it was realized that it is present on

platelets, granulocytes, and lymphocytes. This antigen later proved to be equivalent to Mac (HL-A2) (Balner et al, 1965). $\text{PIGrLy}^{\text{CI}}$ (Shulman et al, 1964) and $\text{PIGrLy}^{\text{FI}}$ (Shulman et al, 1965) were identified, both being HL-A as well (Dausset, 1966; Loucopoulos et al, 1969), and all three antigens can be detected on platelets by C'F only.

The Ko^{a} and Ko^{b} antigens were next identified by van der Weerdt in 1961 (cited by Shulman et al, 1964). They are detected by platelet agglutination only. The PI^{EI} system was recognized in 1962 (Shulman et al, 1964). PI^{EI} and PI^{E2} react by C'F only. All of the above antigens, and their frequencies, are shown on Table 3 of Appendix A.

All of the platelet antibodies described are IgG molecules and thus can cross the placental barrier. Unlike HL-A antibodies, these platelet-specific ones are not absorbed by placental tissue and thus can cause congenital thrombocytopenia. Shulman found PI^{AI} to be the most immunogenic platelet-specific antigen, either in pregnancy or in response to blood transfusion (1964). However, the antigens limited to platelets apparently do not play an important role in human transplantation (Dausset and Rapaport, 1966); for PI^{AI} this is perhaps because of the frequency of the antigen. Besides these platelet-specific antigens, most antigens on platelets are HL-A antigens, common to lymphocytes, granulocytes, kidney cells, etc. (Svejgaard and Kissmeyer-Nielsen, 1970).

Shulman et al also described an antigen specific to lymphocytes, Ly^{DI} (1964). This has a population frequency of 36%, but no genetic family studies have been performed. No other lymphocyte-specific antigens have been reported.

Lalezari has described three granulocyte (or neutrophil)-

specific antigens, implicated in cases of neonatal neutropenia. The first two have been named NAI (1966a), and NBI (1966b), and antibodies against them were shown to be primarily IgG, capable of crossing the placenta. Most of the antibodies to these antigens were produced in multiparous women whose children had neonatal neutropenia, though at least one was produced by active immunization (Lalezari, 1966a).

Significant neutropenia occurs only in cases where neutrophil-specific antigens are involved. Antibodies to leukocyte antigens with wider distribution will be absorbed by various tissues, especially the placenta in a pregnancy-sensitised mother, with a negligible effect on the neutrophils of the fetus (Lalezari, 1966a). Recently Lalezari has described a third neutrophil-specific antigen, NCI (1970), which appears to have a high frequency and is distinct from NAI and NBI.

An important finding made recently is that the HL-A typing of kidney cells differs from that of lymphocytes. Deplanque et al found quantitative differences (1969) indicating that antigens important in transplantation may escape detection. It is not established that the kidney cells possess unique histocompatibility markers. It may be that the immune adherence test (IAT) used with the kidney cells is more sensitive than lymphocytotoxicity. Pierce and Hume have also found IAT more sensitive in detecting antibodies after immunization by a kidney transplant (1971). However, in comparing the results with standard tissue typing sera, the two tests give very similar results. Rolley et al found discrepancies between lymphocytes and kidney cells also. However, since absorption with leukocytes removed the antibody when only mixed agglutination (similar to IAT) with kidney cells was positive, the antibody is not kidney-specific (1969).

B. Tissue Typing Methods and the Antigens,
HL-A and Non-HL-A, Which They Detect.

1. Leukoagglutination and Lymphocytotoxicity

These most commonly used methods of antibody detection have already been mentioned. Leukoagglutination led to the establishment of the first antigenic specificities, but it has become a less popular method for reasons which may turn out to have been less valid than originally supposed. Terasaki called it simple but deceptive, with agglutination being a phenomenon influenced by too many variables. Some of these variations of technique are less with defibrinated blood, which is platelet-free, giving fewer false positives than with EDTA as anticoagulant (Terasaki and Singal, 1969).

Lymphocytotoxicity is the most widely used method now. The two main techniques are the trypan blue exclusion of Amos (1965) or Kissmeyer-Nielsen and Kjerbye (1968), and the eosin-formaldehyde method of Terasaki and McClelland (1964a).

Payne et al tested 11 leukocyte antisera by cytotoxicity and agglutination, concluding that the assays recognize approximately the same spectrum of known specificities (1967). However, Zmijewski and Amos concluded, from a comparison of 66 sera, that antibodies capable of detecting the products of two allelic genes may be present in the same serum - one reactive by agglutination, the other by cytotoxicity (1966).

In the search for non-HL-A loci, Mittal et al, in testing 118 selected antisera, found that antisera reactive in cytotoxicity tests against non-HL-A antigens are quite rare (1969). Any serum reaction discrepancies they found could be accounted for by technical variation. However, the same group later reported evidence in cytotoxic sera

of non-HL-A antibodies which they called HL-B antibodies (Singal et al, 1970). They found some of these HL-B antisera to differentiate between HL-A identical siblings.

2. Complement Fixation, Using Platelets

Shulman's complement fixation method (C'F) for platelets (1962) has been modified and used by Dausset (Colombani et al, 1968), Svejgaard and Kissmeyer-Nielsen (1970), van der Weerd (cited by Colombani et al, 1968) and their co-workers. It has also been modified for use with lymphocytes (Loucopoulos et al, 1969). C'F detects both platelet-specific antigens and HL-A antigens on platelets. Svejgaard and Kissmeyer-Nielsen compared lymphocytotoxicity and platelet C'F in 1098 individuals with 396 sera of various HL-A specificities (1970). They found that most specificities were identical by both methods, but C'F may be less sensitive than cytotoxicity. They believe that if more C'F antibodies are found, the technique could replace cytotoxicity. Its advantages so far are that the platelets are easily prepared in a pure state and can be kept at 4°C for several months.

3. Mixed Agglutination

The mixed agglutination technique was introduced to transplantation studies by Milgrom et al (1965). Similar to this is the immune adherence test introduced by Melief et al (1967). The methods may be more sensitive than cytotoxicity in that they detect antibody to kidney cells before cytotoxic antibodies are evident to lymphocytes. This could also be due to a lag in cytotoxic antibody production (Pierce and Hume, 1971). De Planque et al claim that the techniques do not detect antigens not found on lymphocytes, but kidney cells appear to be more richly endowed with histocompatibility antigens (1971). There is no

evidence that the tests detect other than HL-A antigens.

4. Mixed Lymphocyte Culture (MLC) and its Unidirectional Modification (UMLC)

Another method used in tissue typing is the mixed lymphocyte culture (MLC), where incompatibilities are evidenced by a stimulation of lymphoid blastogenesis. Bach has shown that in a unidirectional culture (UMLC), antigen identity at the HL-A locus can be detected (Bach and Amos, 1967) and antigenic disparity can be quantitated (Bach et al, 1969). There is no stimulation with HL-A identical siblings. With a two-allele difference there is a high degree of stimulation, and with one-allele difference there is an intermediate degree. However, Bach et al (1969) and Yunis et al (1971) later found rare instances of MLC stimulation between cells of HL-A identical siblings, and Rudolph et al (1972) report stimulation between HL-A identical parent and sibling cells. Possible explanations are that non-HL-A antigens are present; the HL-A system is complex, with unrecognized antigenic heterogeneity (Koch et al, 1971); recombination occurred with no detectable antigenic markers on the parts of the chromosomes which were different (Bach et al, 1969); or a separate MLC locus may be present (Koch et al, 1971; Rudolph et al, 1972; Yunis et al, 1971). The above also explain why unrelated HL-A identical individuals usually stimulate the MLC reaction. Only a low percentage are non-stimulatory, such as the 2/9 pairs found by Koch et al (1971) and the 10% of such pairs found non-stimulatory by Sengar et al (1971). The latter found new HL-A specificities distinguished between some of the pairs and others could be explained by subdivisions of known HL-A antigens. Johnston and Bashir found 13 pairs of HL-A identical unrelated individuals to be stimulatory (1971) and supported existence of an MLC locus. Dupont

reported that MLC reactivity is primarily connected with the second sublocus of HL-A - either elicited by the antigens themselves, or an MLC locus closely linked to the second sublocus (Dupont et al, 1971).

If the time required to perform the MLC test could be shortened, it would become more important in transplantation. It quantitates antigenic differences in unrelated as well as related individuals, even though it is yet to be shown, in unrelated individuals, that there is close correlation between MLC and the clinical course of renal allografts.

5. Leukocyte Capillary Agglutination

Capillary agglutination was introduced in a human system by Thompson et al (1968). They developed the technique as a more sensitive assay for leukoagglutinins, since leukoagglutination is variable, and there are many discrepancies between lymphocytotoxicity and leukoagglutination, either because of insensitivity or detection of different antigens (1968).

In 1970, Thompson et al reported the comparison of reactions of 10 cytotoxically monospecific sera by capillary agglutination (CAT), cytotoxicity (CYT), and leukoagglutination (AGG), with two cell types. They found CYT and AGG were positive together only once, therefore may be detecting different antigens. Whenever either of these was positive, and in some instances where neither was, CAT was positive. It may be because of sensitivity or detection of different classes of antibodies (Thompson et al, 1970). In a more detailed study of CAT and CYT, they tested 33 cells typed with 14 antisera monospecific for HL-A. CAT reacted positively twice as often as did CYT for most of the sera, indicating the sera were heterogeneous with respect to CAT antibodies (1970) or that CAT is more sensitive.

Thompson et al have also suggested that CAT is detecting non-HL-A specificities. Several families were studied for their reactions by CYT, CAT and MLC (1970). Among MLC non-stimulatory sibling pairs, there was one pair for which there were some discrepancies in CAT reactions but total agreement for CYT reactions. Thus, not only were CAT and CYT discrepant on individual cells, but CAT was also discrepant between cells of an MLC negative pair. In a further report, sera reactions with cells of known HL-A, NAI, NBI and NCI, and 5a and 5b showed no relation to the CAT reactions (1971). Furthermore, the study of a serum that was CYT negative but CAT positive showed a segregation independent of HL-A in a family study (1971). This is shown on Figure 1.

6. Thermal Dependency in Leukoagglutination and Lymphocytotoxicity

Studies of cold hemagglutinins were extended by Lalezari and Murphy to study cold-reacting leukoagglutinins using EDTA agglutination (1968). They found mixed agglutination of leukocytes and red cells, probably representing similarly reacting antigenic sites on both cell types. These could be the I-i determinants, as Shumak et al (1971) confirmed that the I-i antigens of human adult red cells and cord red cells, respectively, are easily detected on lymphocytes of both adults and infants. Anti-i and anti-I sera were found to have lymphocytotoxic activity both at 25°C and when incubation was 30 minutes at 4°C followed by 30 minutes at 25°C.

However, in Lalezari's work, pure leukocyte suspensions agglutinate with cold leukoagglutinins, some of which cannot be absorbed by red cells, indicating other antigenic factors. There are three types of thermal dependency for leukoagglutinins: 1) Those reacting at 4°C with no activity at higher temperatures, 2) Those reacting best at 25°C

FIGURE 1

RELATION OF A CAPILLARY AGGLUTINATING (CAT) SERUM
TO HL-A HAPLOTYPE SEGREGATION WITHIN A FAMILY

	Mother		Father	
HL-A Haplotypes	ab		cd	
CAT Response	+		-	
Number of Siblings	ac	bc	bd	ad
	4	3	1 1	3
CAT Response	+	+	+	-
			-	-

Source: Thompson, J.S., Severson, C.D., Parmely, M.J., Marmonstein, B.L., and Simmons, A.: Pulmonary "hypersensitivity" reactions induced by transfusion of non-HL-A leukoagglutinins. N. Eng. J. Med. 284: 1120-1125, 1971. Table 3, p. 1124.

but losing activity at 4°C and 37°C, 3) Warm leukocyte antigens, reacting at 37°C, with no activity at lower temperatures or at 56°C. Lalezari believes that cold leucoagglutinins can offer an explanation for various types of leukopenia and thrombocytopenia, and also that they may interfere in the study of warm leukocyte antibodies if the reaction mixtures are left at room temperature (Lalezari and Murphy, 1968).

More recently, cold autolymphocytotoxins were found in infectious mononucleosis, rubella and measles (Mottironi et al, 1970), rheumatoid arthritis, and lupus (Terasaki et al, 1970b). The explanation of their presence is unclear - one of the possible reasons is antibody cross-reactions with microbial and lymphocyte transplantation antigens. These antibodies were also detected in multiparous women and kidney transplant recipients (Naito et al, 1971). They are principally 19s (IgM), whereas most HL-A antibodies are IgG, and their specificity is unrelated to HL-A specificities.

7. Recent Innovations

Cytolysis in agar gel was recently described by Juji et al (in press, 1972), and was performed with transplantation sera and renal cells. It involves the diffusion of antiserum and complement through agar containing the cells - resulting in lytic zones in the cell layer. This technique correlates with mixed agglutination, but is more convenient.

Grothaus et al found that human lymphocytes treated with neuraminidase are more susceptible to lysis by specific HL-A antisera and sera non-reactive with untreated cells, in the cytotoxicity test (1971). They suggest that the antigens are made more accessible to antibodies, increasing the binding of antibody. Work is progressing by these investi-

gators in collaboration with Thompson to see if these more sensitive reactions correlate with reactions obtained by the capillary agglutination test. However, Ray et al claim that neuraminidase increases cell sensitivity to complement in the absence of antibody (1971).

Lucas et al have reported a method to detect subliminal sensitization responsible for early transplant failure (1970). The assay is based on the inhibition of binding of radioiodine-labelled anti-HL-A IgG by recipient serum. The titre of antibody detected correlated with the severity of early renal failure. The assay is 50-fold more sensitive than cytotoxicity: patients with binding antibody titre of 2^7 (1:128) or greater had detectable cytotoxic antibody.

It is essential that techniques such as these continue to be developed, so that the most useful methods are put into practice for crossmatching and antibody detection.

C. Classes of Immunoglobulins

In this review, every aspect of the immune response cannot be dealt with. The differentiation of T and B cells from stem cells, the role of thymic migrations and thymic hormones, the concept of T and B cell collaboration in induction of certain types of immune response, the possible role of the macrophage, the feedback mechanisms whereby the immune response is regulated; all these important aspects of immune induction and its control must be accepted. The concern of this thesis is various types of antibody reaction following HL-A exposure, and their significance, so it is to the nature of antibodies that attention will now be directed.

Tiselius, in 1936, first recognized the gamma globulins as a

distinct group of serum proteins. He later found that this fraction consists of five distinct immune globulins: IgG, IgM, IgA, IgD, and IgE (cited by Weiser et al, 1969). All humoral antibody has been found to have the basic structure of two heavy chains and two light chains covalently linked by interchain disulphide bonds, giving the familiar IgG structure (Cohen and Milstein, 1967).

It is the Fab fragment, obtained after papain digestion, which possesses the antigen-binding sites at its N-terminal end. Thus, each IgG molecule has two monospecific antibody-combining sites. The specificities are determined by the amino acid sequence in the variable segments of the heavy chains. That of the light chains enhances the binding activity and determines the secondary and tertiary folding. The Fc fragment determines all other properties specific to each class. Accordingly, it is the heavy chains which differ antigenically as shown by immunoelectrophoresis and this is the basis of the class differentiation. There are also subclasses, based on these electrophoretic patterns. Table 4 of Appendix A gives the current knowledge of the three main immunoglobulin classes with antibody activity (Cohen and Milstein, 1967; Weiser et al, 1969).

There has been some genetic typing of immunoglobulins (Weiser et al, 1969). The genetically controlled intra-species characteristics are "allotypes". The Gm locus governs the amino acid sequence of the Fc fragment of the heavy chain of IgG. At least twenty variants are known for this locus. The InV locus determines light chain structure of all immunoglobulins. There are at least five variations of this.

In the immune response IgM is the first to respond. It then decreases as IgG and IgA increase (Weiser et al, 1969). Separation of these

antibody classes and some correlation with each antibody-detection technique has been achieved. Metzgar and Seigler (1967) fractionated human antisera, containing leukoagglutinating and cytotoxic antibodies, on Biogel P-300 polyacrylamide chromatographic columns. They found pure IgG had agglutinating activity; the same concentrations were not cytotoxic but possibly they were too low. IgM from some antisera agglutinated some cell types but showed no cytotoxic activity. Previous studies by Engelfriet and Britten (1965) and by Walford et al (1965) attributed cytotoxic activity to IgG. Walford only had fractions of "crude globulin", $\beta_2\gamma_1$ -globulin, and γ_2 -globulin. Stocker et al (1969) showed that IgG was cytotoxic in sera from subjects immunized by multiple transfusions or pregnancy. In sera of patients who had rejected renal allografts, cytotoxicity was found in IgM fractions (Sephadex G-200) either alone or in conjunction with IgG activity (column chromatography on DEAE cellulose). Johnson et al subsequently fractionated cytotoxic sera from cardiac and renal transplant patients, most of them by sucrose density gradient ultracentrifugation (1970). In the cardiac group (30 sera from 7 patients), lymphocytotoxic activity was in the IgM region exclusively. However, in the renal patients (3 sera from 2 patients), this activity was only in the IgG (7s) region. They hypothesize that the 7s antibodies were absorbed by the heart grafts, or that the 7s lymphocytotoxins are blocked by serum substances.

Ahrons and Glavind-Kristensen separated IgG and IgM by sucrose density ultracentrifugation (1971) and they found that leukoagglutinating and cytotoxic antibodies were in the IgG and IgM fractions in sera from pregnant women, transfused patients and recipients of skin grafts. Twenty-two of 23 sera had IgG activity. In two patients with rejected

kidney grafts, only IgG was active, in contrast to Stocker's findings.

Voisin et al (1969), using curtain or Pevikon block electrophoresis of mouse serum, showed that antibody causing cytotoxicity and inhibiting tumour growth is a slowly migrating IgG γ_2 , and is complement fixing. IgM plays little or no role. They also found that in vivo enhancement correlated with γ_1 IgG which is fast migrating and non-complement fixing. Takasugi and Hildemann (1969) however, found IgG γ_2 (isolated by ion exchange chromatography) responsible for enhancement of mouse tumours and IgM (Sephadex G-200) implicated in cytotoxicity and inhibition of tumour growth. Mullen and Hildemann (1971) obtained similar results in rat kidney transplants. Hellström and Hellström have indicated that IgG might be their "blocking" or enhancing factor (Sjögren et al, 1971), and Miller et al (1971) found IgG specifically caused MLC inhibition (possibly enhancement).

Table I summarizes these results. There is no general trend, and both IgG and IgM have been implicated in cytotoxicity and agglutination, while only IgG has been named responsible for enhancement. It may be mentioned, at this point, that there seems to be no way of separating pure IgG from IgG-soluble antigen complexes; this is important in some more recent concepts of mechanisms whereby enhancement or immunoregulation carry out their effects. Central mechanisms, in contrast to peripheral ones, invoke antigen complexed IgG as the moiety that acts centrally.

TABLE I

THE ANTIBODY CLASSES IMPLICATED IN CYTOTOXICITY, LEUKO-
AGGLUTINATION AND ENHANCEMENT, BY VARIOUS WORKERS

Investigators	Cytotoxicity		Agglutination		Enhancement	
	IgG	IgM	IgG	IgM	IgG	IgM
Metzgar & Seigler (1967)	-	-	+	+	ND*	ND
			(most)			
Englefriet & Britten (1965) & Walford et al (1965)	+	-	ND	ND	ND	ND
Stocker et al (1969)	+	+¶	ND	ND	ND	ND
Johnson et al (1970)	+¶	+§	ND	ND	ND	ND
Ahrns and Glavind- Kristensen (1971)	+¶	+ (some)	+	+	ND	ND
Voisin et al (1969)	+Y ₂	-	ND	ND	+Y ₁	-
Takasugi & Hildemann (1969)	-	+	ND	ND	+Y ₂	-
Miller & Hildemann (1971)	-	+	ND	ND	+	-
Sjögren et al (1971)	ND	ND	ND	ND	+	-
Miller et al (1971)	ND	ND	ND	ND	+	ND

*

ND - Not done

¶ - Renal transplant patients

§ - Cardiac patients

D. Histocompatibility Antigens and Antibodies in Transplantation

I. Preformed Antibody

Hyperacute rejection will occur if a transplant recipient has previously developed cytotoxic antibodies to the HL-A antigens of the donor. The phenomenon was first described by Kissmeyer-Nielsen et al (1966), and later by others (Terasaki et al, 1968; Starzl et al, 1968; Jeannet et al, 1970). Terasaki et al tested seven patients who had rejected hyperacutely (1968). All had lymphocytotoxic antibodies before transplantation, and of five tested, all were positive with the donor's cells. Six of the patients were multiparous women, differing antigenically from their husbands. Each donor shared an incompatible antigen with the husband. The seventh patient was given a kidney from a donor sharing antigens with her first kidney graft, which had been rejected. A later report from these investigators showed immediate failure in 24 of 30 kidneys transplanted across a positive crossmatch (Patel and Terasaki, 1969). Jeannet et al (1970) also cited 10 cases where the presence of antibodies to the donor antigens resulted in immediate rejection.

A further finding by Terasaki et al (1971) is that renal allograft recipients with preformed lymphocytotoxins not only have 8% incidence of hyperacute rejection despite a negative crossmatch, but those that function also have a poor long-term result; this latter is even more marked in unrelated second allografts. Second graft recipients without antibodies had a higher survival than first graft recipients, indicating non-cytotoxic enhancing antibodies. The poorer outcome with antibody

is also shown by Patel et al (1971a). Preformed antibodies play the same role in skin grafting. Dausset and Rapaport immunized individuals with group-specific antigens (1966). Subsequent incompatible skin grafts underwent either accelerated or hyperacute (white graft) rejection. They also reported that compatible grafts survive longer, in pre-immunized individuals, with respect to the antigens HL-A2 (8a), HL-A7 (6b, 7c), 4a and 4b (1965b). Leukoagglutinating or cytotoxic antibodies have also been implicated in febrile transfusion reactions (Payne et al, 1964).

Anti-HL-A antibodies can develop through multiple blood transfusions, a previous graft, or pregnancy. Among 218 prospective kidney recipients undergoing hemodialysis, Terasaki et al found 30% had preformed cytotoxic antibodies (1968); presumably this is due to blood transfusions, though the frequency of transfusion was not available to him. In 1969, this percentage had dropped to 19.2% among 681 patients (Terasaki and Singal, 1969). Female recipients had 25.7% and males had 15%. Among 1715 recipients in 1971 (Terasaki et al, 1971) 16.7% had antibodies, 24.2% in females and 13.5% in males. In the national pool at this time, there was a 25.5% incidence. In 1972, these investigators state that the value of 20-25% is a general finding regardless of the duration of hemodialysis (Opelz et al, 1972). However, some patients who continue on hemodialysis but are no longer being transfused, lose their antibody with time, so that the accumulated frequency is higher (up to 60%). Data from Oh et al (1972) agree with this, with 15-20% of patients developing antibody after each six month interval on dialysis - the accumulated frequency at 1½ years being 59.6%.

Among patients who have rejected first or second transplants,

the incidence of antibody is as high as 54.8% among 51 patients (Terasaki et al, 1971).

In 1958, Payne and Rolfs found leukoagglutinins in 17% of 144 multipara (four or more pregnancies). Further reports from Payne (1962) and van Rood et al (1959) show that 24% and 15% respectively develop leukoagglutinins, and Payne found that the antibodies of 16 of 31 women were still detectable three years after delivery (1962). More recently, Zmijewski et al found a frequency of 25.06% among 1584 women (1967). They found the incidence was proportional to the number of pregnancies, with 15% of primigravida and 33.15% of multipara having antibody. Terasaki et al confirm this increase with parity, with respect to cytotoxic antibodies (1970a). A recent report reveals that the antibodies developing during pregnancy are not necessarily harmful to the graft outcome (Beleil et al, in press).

A higher percentage of antibodies in primigravida was found by Ahrons, at the University of Aarhus, Denmark (1971). In a series of 50 women, 10% had cytotoxic antibodies by the 24-34th week. This increased to 22% at delivery and 30% at 4 weeks postpartum. By leukoagglutination, the figures were 20% to 30% to 34%. Ahrons gives reasons for his high percentages: 1) He used a highly selected leukocyte panel representing 17 antigens; 2) Acetic acid, which others add to the reaction mixture in agglutination, reduces the titre three-fold.

The pre-transplant lymphocytotoxic crossmatch is essential with such high antibody frequencies in all these groups of prospective kidney recipients. There have only been a few instances where transplantation has been successful with a positive lymphocytotoxic crossmatch (Patel

et al, 1971b; Morris et al, 1969c]. In the review above and in the data presented in this thesis, there are many instances of discrepancy between lymphocytotoxicity (which may be negative) and leucoagglutination or leukocyte capillary agglutination (either or both of which may be positive). In such circumstances should the pre-transplant crossmatch be considered positive? The literature is obscure on this point.

2. Undetected Pre-sensitization

There have been several cases reported of hyperacute rejection in the face of a negative cytotoxic crossmatch (Starzl et al, 1968; Morris et al, 1969b). These are considered to be false negative reactions, or are termed "Schwartzman reactions", where antibody-antigen interactions might not be involved. J.C. Pierce et al reported an increase of these hyperacute rejections among patients with multiple renal allotransplants (1971). One possible explanation is that there may be sensitization to renal-specific antigens that cannot be picked up by the cytotoxic test. In some of the above patients with previous transplants, there was a positive pre-transplant crossmatch with the donor kidney cells, by mixed agglutination or immune adherence. It was also shown that with immunization by a kidney transplant, antibodies, as detected by lymphocytotoxicity, lagged behind antibodies detected by immune adherence against kidney cells (Pierce et al, 1971). Pierce and Hume (1971) state it is important to exclude potential transplants with major HL-A mismatches with previously rejected kidneys. We have also reported data to support this (Dosssetor and Olson, 1972).

Subliminal sensitization responsible for early transplant failure was also detected by Lucas et al (1970), by inhibition of binding of

radioiodine-labelled anti-HL-A IgG. The titre of the antibody detected correlated with the severity of early renal failure among recipients who had no previous transplants.

Casely et al also showed presentization of non-transplanted patients (1971). They studied patients on hemodialysis, receiving many blood transfusions, and found there was higher antibody development when whole blood is given, as opposed to leukocyte-poor blood. Reactivity varied with time, and cytotoxic antibodies against specific cells disappeared with time. They recommend crossmatching with all serum samples available, to avoid a "second set" rejection. Opelz et al also report the loss of antibody with time in these patients (1972). The percent of cytotoxically negative pre-sensitized patients is as high as 40% after 1½ years on dialysis.

Dossetor et al (1967), Opelz et al (1972), and Tremann et al (1971) have all shown a lack of sensitization of potential renal recipients by multiple transfusions. Opelz et al show that long-term dialysis patients who do not form antibody may also be less responsive to HL-A incompatible kidney transplants and have a higher survival rate than those who do form antibody. One case of a latent antibody after transplantation and immunosuppression has been reported by Dossetor (1970). The patient, with chronic renal failure and uremia, had developed a wide spectrum of HL-A antibodies during hemodialysis, presumably due to the multiple whole blood transfusions given. He received a kidney, with a negative donor lymphocyte-recipient serum crossmatch. During the subsequent five months there were a number of severe rejections, and he was given intensive immunosuppression, but this was unsuccessful, and the

kidney had to be removed. It was then noted that cytotoxic antibodies were no longer present in his serum. For the next five months of hemodialysis he was given only buffy coat free blood, which has much reduced numbers of white cells, and his antibody remained absent. However, his sensitization was still present, though latent: when given a whole blood transfusion from his mother, a potential live donor, antibody specifically against her lymphocytes immediately developed. His pre-transplant serum was also found to have been cytotoxic to his mother's cells. Subsequent further whole blood caused the reappearance of his full spectrum of antibodies.

3. Correlation of Tissue Typing and Graft Success

The role of HL-A antigens where there are no preformed antibodies is difficult to assess. Ceppellini et al (1966) showed a significant difference for the following groups of ABO compatible skin graft donors: 12.1 days for unrelated donors, 14.1 days for parents, and 16.7 days for siblings. Amos et al furthered this to show that HL-A identical siblings had a prolonged skin graft survival of 24.9 days. Siblings differing at one allele, and parent to child, had intermediate prolongation of 15 days. Siblings differing at both alleles had as short a survival of skin grafts as unrelated individuals (11 days) (1969). Koch et al showed that HL-A identical unrelated individuals had a prolonged skin graft survival of 11.4 days as compared to 10 days for unrelated non-identical persons, whereas HL-A identical siblings gave 18.9 days in this series (1971).

In kidney transplantation there is also a significant effect in survival depending on the genetic relationships involved, as shown in

the latest report of the Advisory Committee to the Renal Transplant Registry in Chicago (1972). Their report, up to February, 1972, shows overall survival of kidney transplant recipients (without regard to HL-A antigen disparity per se) at one and at two years, respectively, as follows: unrelated (cadaver) donor 67% and 65%, parent donor 88% and 83%, sibling donor 82% and 86%. The percent functioning at these times are: cadaver donor 47% and 46%, parent donor 66% and 67%, and sibling donor 73% and 79% (Table I, Appendix A). Singal et al studied live related kidney transplants for correlation between antigen disparity and survival (1969). Within the families, when there was no mismatch (and we may presume HL-A identity) the four year sibling survival was 92%. When there was marked mismatch between siblings (and we may assume both one and two haplotype differences were incompatible) the four year survival was 43%. With parents (who can only differ by one haplotype), the better matched had 81% and the worse matched had 68% four year survival. Thus the results by antigen analysis (and known haplotype distribution) fit well within the prognostic restrictions of the HL-A system.

With unrelated donors, there is much less agreement that prognosis is related to HL-A typing. Van Rood (1969, 1971), Batchelor (1971), Morris (1970, 1971), Kissmeyer-Nielsen (1970, 1971) and Kountz (Perkins et al, 1971) and their co-workers claim that there is; but Terasaki (Mickey et al, 1971; Halgrimson et al, 1971) and Hamburger et al (1971) fail to find support for this viewpoint, though Dausset, who performs the histocompatibility testing for the Hamburger group, finds a correlation if cross-reacting antigens are considered as identities (1971).

In 196 transplants analysed by Terasaki et al (1967), the ratio of survival to non-survival for "matched" was 71:15, and for "mismatched"

was 71:35. In 1969, with the same patients, the ratios were "matched" 66:20, "mismatched" 57:49 (Terasaki and Singal, 1969). The increased significance with time demonstrates the long-term influence of HL-A incompatibility in human transplantation. However, in 1970, at the Third International Conference of Transplantation (Halgrimson et al, 1971), Terasaki states that "while good HL-A matching should be a desirable condition in performing organ transplantation, the HL-A system may only be the tip of an enormous and, as yet, poorly understood biological iceberg".¹ He sees the need for more sophisticated measures of HL-A compatibility for more accurate prediction of outcome (Mickey et al, 1971).

Van Rood et al showed the most clearcut correlation of HL-A typing and graft success, though it was with only 37 patients (1969). They found that the fraction of graft survivals decreases as the number of HL-A differences increases from 0-4, and conclude that leukocyte matching is able to improve the prognosis of a kidney allograft. However, in 66 cadaver donors, Dausset found no significant correlation of HL-A typing with transplant success. When any cross-reactions were considered as identities, there was a striking positive correlation (1971).

The relative strengths of different HL-A antigens is not known. HL-A2 does function as a strong transplant antigen, with renal recipients mismatched for it doing worse than compatible ones (Terasaki and Singal, 1969). HL-A2, HL-A7, 4a and 4b are similarly significant in skin grafts, when the recipient has been pre-immunized (van Rood et al, 1965; Dausset and Rapaport, 1966). Morris et al found 4a, 4b, HL-A2, 3 and 8 incompat-

¹ Halgrimson, C.G., Rapaport, F.T., et al. Net Histocompatibility Ratios (NHR) for Clinical Transplantation. Transpl. Proc. 3(1): p. 144, 1971.

ibility to adversely affect the early course of a kidney graft (1969a). Ward et al extended this to include HL-A1, 5, 7, 12 and W18 as significantly associated with rapid rejection of related and unrelated skin grafts (1970).

All the above instances where a given author claims importance for a particular HL-A antigen must be considered as purely tentative at this time, and not as established fact. Perhaps the strength of a given antigen can only be defined with respect to associated donor antigens and against the background of the different HL-A profiles of the recipients. Also, the HL-A antigens, or combinations of them as antigen profiles, will have to be related to emerging concepts of an MLC locus that may control the immune response. As yet virtually no progress has been made in this area. The most recent attempt is that of Mickey et al, where the appearance of an antibody is related to its frequency of exposure in multiparous women (1972); the most immunogenic antigens were HL-A2, 5, 7, 17 and Te50, the least responsive antigens in a recipient profile were HL-A10, 11, 14 and Te60. Women with HL-A28 did not make anti-HL-A2; women with HL-A2 did not make anti-HL-A28. Similar relationships were shown between HL-A3 and HL-A11, consistent with studies of cross-reactivity. In the case of HL-A1, which absorbs out HL-A11 antibodies, but HL-A11 platelets do not absorb HL-A1 antibodies (Sengar, personal communication), the predicted happened: HL-A1 mothers made no antibodies to HL-A11 husbands but the opposite does not hold. Making adjustment for these factors, the Terasaki group has been able to improve their correlation between typing and kidney function (Mickey et al, 1972), but it is still not very good. Known cross-reactivity has changed since this last paper was published (Figure 2, Appendix A).

4. Enhancing or Inhibitory Antibody

Humoral antibodies against HL-A antigens may protect against cell-mediated immunity to tumours, pregnancy, or allografts (Ceppellini et al, 1971). This in vivo enhancement may be reflected by interference with in vitro reactions. Ceppellini et al have reported the use of the unidirectional mixed lymphocyte culture (UMLC) to explore this problem (1971). They used recipient and donor lymphocytes following multiple transfusions. In all cases there was complete inhibition where the specific donor is the stimulator, with inactivated serum of the recipient added. This is shown to be very sensitive in detecting antibodies not recognized by current serological methods, and is also a good method for studying the interference between humoral antibodies and immunocompetent cells. Gordon et al (1971) found, in rats, that enhancing antibodies were inhibitory to the MLC reaction.

Hattler et al have shown inhibition of UMLC following a successful renal allograft (1971). IgG separated on DEAE columns from the transplant recipient was shown to progressively inhibit the UMLC when donor lymphocytes were used. This IgG had no lymphocytotoxic activity and may serve an immunoregulatory function after transplantation. These workers also showed this specific inhibition in IgG from immune sera following renal transplantation in the dog (Miller et al, 1971). The UMLC inhibition correlated with cytotoxicity, but was a more sensitive detector of antibody, perhaps enhancing antibody. Jenkins and Hancock (1972) and Kasakura (1971) have found that some factor in maternal serum also causes this inhibition of UMLC, with combinations of maternal and paternal lymphocytes. Kasakura found this effect to be non-specific,

possibly being related to the levels of 17-hydroxycorticosteroids during pregnancy. Leventhal et al found that IgG could be the factor responsible for the MLC inhibition (1970).

Terasaki et al have recently presented preliminary evidence for enhancing antibody in multiparous kidney recipients (Beleil et al, in press). Preformed antibodies are found at twice the frequency among females, but there is no difference in survival between male and female recipients, or in females with and without antibodies. In addition, eight recipients with transplants functioning despite a positive cross-match were female. Further to this report, these workers found long-term dialysis patients with no cytotoxic antibodies do much better than those with antibody (Opelz et al, 1972). One hypothesis they offer is that these patients may have enhancing antibodies, inversely related to the presence of cytotoxic antibodies. Cross-reactions with autologous HL-A antigens could also result in no response. Dossetor et al (1967) had previously found that recipients with greater hemodialyses prior to transplantation showed less evidence of rejection. The action of the inhibitory antibody has been studied further in vitro and in vivo in animals. Of the two possible mechanisms - blocking of the peripheral or of the central part of the immune response - the latter has recently begun to gain greater acceptance. McKenzie et al produced enhancement of mouse skin grafts by injection of specific antibody, believing this to be central or afferent inhibition (1971). Guttman and Lindquist produced enhancement in rat kidney grafts, with bone marrow cells. This could be passively transferred by antibody, and involves a central inhibition (1971). Hellström and Hellström showed that patients with actively growing tumour have a factor in their serum which blocks the killing

effect of their own immune lymphocytes. In regression of the tumour, this is lost. In kidney patients, only those doing well have this factor against the lymphocyte effect (G.E. Pierce et al, 1971). Morris and Lucas, however, support the concept of peripheral coating of the graft cells (1971). Labelled IgG was rapidly "fixed" to various organs in kidney-grafted rats.

Hellström et al have since provided evidence that the blocking antibodies may be antigen-antibody complexes (Sjögren et al, 1971). At low pH, with an Amicon filter, they separated a low and high molecular weight fraction. Alone, neither has a blocking effect, while the mixture has a specific blocking effect. They believe the likely "point of action" is the immune lymphocyte, with a temporary blocking of the lymphocytes' reactivity. They have also found a "deblocking" antibody, abrogating the blocking activity, in sera of patients whose tumour has regressed (G.E. Pierce et al, 1971).

The only attempt at induction of human kidney enhancement was by Batchelor et al (1970). They administered Fab and F(ab')₂ from IgG reactive against the donor's cells. At three months, function was good, though some immunosuppression was required. As the donor was only disparate by one haplotype, and as a definite rejection crisis did occur, one cannot claim that this first attempt was successful, but it probably points the way in which clinical transplants will go.

5. Antibodies Developing Post-transplant

a. Cytotoxic or agglutinating antibodies (not always anti-HL-A) - In this thesis, data will not be given for antibodies occurring after transplantation. Most consider this to be a rather infrequent phenomenon.

However, Morris et al found cytotoxic antibodies to be associated with transplant rejections - either acting on the transplant or serving as an indicator of pre-sensitization (1968). Those patients with antibodies had more kidney failure and worse kidney function. Jeannet et al confirmed this (1970), 16 of 28 patients developing antibodies to the donor after transplantation - nine of these being shown by mixed agglutination only. These patients were less well-matched to the donor than those who did not form antibodies, and had poorer progress; 12 of them rejected their transplants or died. Milgrom et al have shown antibodies, as detected by mixed agglutination, are often removed from the circulation by the graft (1966; Williams et al, 1968). Thus, although one might expect that most kidney recipients do form antibodies, they are not necessarily detected.

b. Enhancing or inhibiting antibody - This has been reviewed above. Hattler et al, notably, have shown a non-cytotoxic IgG antibody which inhibits MLC, following renal transplantation, in the human (1971) and the dog (Miller et al, 1971). Hellström's group have found that within the first year after transplantation, most recipients have a blocking factor in their serum, with a good correlation between the blocking activity and a favourable clinical course (G.E. Pierce et al, 1971).

CHAPTER III

METHODS

A. Description of Techniques

1. Lymphocytotoxicity (CYT)

The eosin-formaldehyde method of Terasaki was performed throughout the study (Mittal et al, 1968). The steps are as follows:

a. Lymphocyte preparation - Pure lymphocyte suspensions without granulocytes and red blood cells must be used.

- i. 10 ml of heparinized blood (1000 units of heparin per 10 ml of whole blood) are spun in an International clinical centrifuge model CL at speed #4 for 7 minutes.
- ii The buffy coat is removed and spun in 1 ml Fisher centrifuge tubes for 2 minutes at 3500 g in a Fisher centrifuge, model 59.
- iii. The buffy coat is removed and spun in 0.4 ml Beckman polystyrene tubes for 10 seconds at full speed in a Beckman Spinco Microfuge, model 152.
- iv. This buffy coat is now placed in a Fisher tube containing 0.25 ml of anti-AB serum or anti-H (from *Ulex europeaeus* seeds) and rotated gently until the red cells are agglutinated.
- v. The suspension is centrifuged at 1000 g for 2-3 seconds in the Fisher centrifuge to remove the agglutinated red cells.
- vi. The supernatant is removed and centrifuged in a Beckman tube at full speed for ten seconds, to agglutinate any remaining red cells.
- vii. The supernatant is discarded and the cells are suspended in McCoy's medium 5a, using a pasteur pipette to free the leukocytes from the clumped cells.

- viii. The cells are spun at one-third speed in the Beckman centrifuge ("quick spin") for two seconds, to remove residual red cells.
- ix. The supernatant is spun at full speed in the Beckman centrifuge for ten seconds, to wash out the anti-AB serum. The sedimented leukocytes are suspended in 0.1 ml of normal human AB serum and 0.1 ml of McCoy's medium.
- x. The cell suspension is given a "quick spin" for two seconds, and the supernatant is placed on a column of 0.2 mm diameter glass beads contained in a nylon plugged plastic drinking straw, and incubated at 37°C for three minutes. (The beads have been pre-coated with 1% gum arabic, and dried.)
- xi. The lymphocytes are flushed out with McCoy's medium into a Fisher tube.
- xii. The suspension is centrifuged at 4000 g for two minutes, the cell button is resuspended in 0.4 ml of McCoy's medium with 30% fetal calf serum, and the lymphocytes are counted and adjusted to 2×10^6 cells per ml.

b. Setting up the tests

- i. Sera are dispensed into disposable polystyrene microtitre trays (Falcon #3034), with 60 wells each. The wells are filled with mineral oil, to prevent evaporation, and 1 λ of serum is added to each well, using a multiple repeating dispenser with 50 λ Hamilton syringes. The trays are stored at -60°C until use.
- ii. The lymphocyte suspension is mixed thoroughly and 1 λ added to each well with a 50 λ Hamilton syringe.
- iii. Cells and sera are mixed, and incubated at room temperature for 30 minutes.
- iv. 5 λ of rabbit complement is added to each well with a 250 λ Hamilton syringe, for a further incubation of 60 minutes.
- v. 3 λ of aqueous eosin is added to each well with a multiple needle dispenser on a Hamilton syringe.

- vi. After 3 minutes 8 λ of formaldehyde is added in the same way.
- vii. A 50 x 75 mm microscope slide is lowered onto the wells to flatten the top of the droplets.

c. Reading the tests

- i. The tests are read with an inverted phase contrast microscope using a 10 \times objective. Living lymphocytes are small and refractile, and dead ones are larger and stained with eosin.
- ii. The following code was used:
 - 1 = negative, with same viability as controls
 - 2 = doubtful negative, with slight increase in dead cells
 - 4 = doubtful positive, with a further increase in dead cells, but less than 50%
 - 6 = positive, with 50-80% cell death
 - 8 = strong positive, with 80-100% cell death
 - 0 = no reading can be made

d. Using the test

To type lymphocytes, typing trays from Terasaki's laboratory in California were used, as well as trays made from sera obtained by our own laboratory. All of the recognized HL-A specificities were present, and most of the W or Te specificities. Lymphocytes for which all four antigens are detected are said to have a "full house". Those with only two or three antigens either possess undetectable antigens or have one or both homozygous antigens.

To detect cell specific but HL-A unspecified antibody monthly screens with sera from patients on hemodialysis, or sera from multipara or postmenopausal multipara before and after transfusion, were done. The sera were tested, in triplicate, against a panel of five more or less constant cell types.

To determine an antibody specificity, a serum was tested

against a large panel of lymphocytes of known HL-A profile and any mono-specific sera, or those with 2 or 3 well-defined specificities, could be pinpointed.

To do the pre-transplant crossmatch test, lymphocytes of prospective donors were tested against sera from all possible recipients, the sera being in 1, 2, and 3 λ quantities.

2. Leukoagglutination (AGG)

Payne's technique, using defibrinated blood (1957) has been used with few modifications.

a. Leukocyte preparation

- i. 10 ml of whole blood are placed in a plastic cup with six glass beads of 6 mm diameter, and rotated at 180 rpm on a Yankee Variable Speed Rotator (Clay and Adams) for 15 minutes.
- ii. The defibrinated blood is mixed with 2.5 ml of Plasmagel or 4% polyvinylpyrrolidone (PVP: M.W. 40,000) in saline, in a disposable plastic test tube, 15 x 150 mm.
- iii. The mixture is allowed to sediment for about 30 minutes at 37°C at a 45° angle. The PVP or Plasmagel causes rouleaux formation of the erythrocytes, accelerating the rate of sedimentation.
- iv. The supernatant is removed as a leukocyte suspension. The optimum cell concentration is 2500-3500 cells per cu. mm (Payne, 1957). Actual counts ranged from 2000 to 6000 cells per cu mm. The percentage of lymphocytes varied from 60-75%.

b. Setting up the tests

- i. Sera are dispensed into clean glass test tubes, 10 x 75 mm, 1 drop from a Pasteur pipette (about 0.05 ml) per tube. All sera are heat-inactivated before use, at 56°C for 30 minutes.

- ii. The cells are added, 1 drop per tube; the suspension is rotated gently, and incubated at 37°C for 1¼ to 1½ hours.
- iii. Any erythrocytes in the mixture are lysed by the addition of 2 drops (0.1 ml) of 6% acetic acid.
- iv. The suspensions are mixed, and each is placed in a thin layer on a microscope slide.

c. Reading the tests

The original procedure was to place the suspension on a slide and read it wet. However, there is increased accuracy and convenience with the following improvement, introduced by Haystead (1968).

- i. The suspensions are allowed to dry on the slides.
- ii. The slides are stained with Wright's stain (2 minutes with the stain, 1 minute with Wright's buffer), rinsed with cold tap water and dried.
- iii. The dried slides are covered with mineral oil (for ease in reading) and examined microscopically with a 10× objective. The results are recorded as follows:

- = negative, no agglutination
- + = positive, with small clumps and many free cells
- ++ = positive, with larger clumps and fewer free cells
- +++ = positive, with large clumps and some free cells
- ++++ = very strongly positive, with large clumps and no free cells

d. Using the test

Leukoagglutination was used only to detect an unspecified antibody, in the same situations as lymphocytotoxicity. Originally each test was performed only once. However, the test was discontinued for several months, and when put into practice again each test was at least duplicated. In any crossmatches, the sera were tested in 1, 2, and 3

drop quantities (or approximately 0.05, 0.1 and 0.15 ml).

3. Leukocyte Capillary Agglutination Test (CAT)

This is performed using the technique of Thompson et al (1968).

a. Leukocyte preparation

- i. 10 ml of whole blood are collected in 1 ml of 5% disodium ethylenediamine tetracetate (EDTA) in saline.
- ii. The blood is mixed with 2.5 ml of Plasmagel (Laboratoire Roger Bellon, Neuilly, France) and allowed to sediment for about 30 minutes at 37°C at a 45° angle.
- iii. The supernatant is centrifuged at 220 g (1000 rpm in an International centrifuge model PR-6) for 15 minutes.
- iv. The supernatant is discarded, 10 ml of shocking solution is added, vortex-mixed, and allowed to stand for 60 seconds (for red cell lysis).
- v. 1 ml of neutralizing solution is added and vortex-mixed.
- vi. The leukocyte suspension is centrifuged at 185 g (850 rpm in an International centrifuge, model PR-6) for 5 minutes.
- vii. The cell button is resuspended in 1.5 ml of gelatin, EDTA, phosphate, saline buffer (GEPS), and layered over a column of 6% dextran in GEPS in a 7 x 100 mm glass tube. This is centrifuged at 118 g (700 rpm) for ten minutes (or 66 g for 15 minutes).
- viii. The supernatant (with platelets and cell debris) is discarded and sedimented leukocytes washed in 2 ml of GEPS.
- ix. The suspension is centrifuged at 118 g for 4 minutes; the cells resuspended in 1 ml of GEPS with 1:8 AB serum.
- x. The leukocytes are counted and adjusted to $20-30 \times 10^6$ cells per ml. The percentage of lymphocytes, in a series of 41 preparations, ranged from 20-50%, with an average of 28%.

b. Setting up the tests

- i. Sera are heat-inactivated at 56°C for 30 minutes and centrifuged in the Beckman Spinco Microfuge or the Model 59 Fisher centrifuge at full speed for a minimum of 5 minutes.
- ii. Disposable polystyrene microtest trays with 96 wells (Cook Engineering #220-24A) are used, with each serum added to 2 wells in 10 λ quantities with a 50 λ syringe. The trays are covered with 3 $\frac{1}{4}$ " wide scotch tape and stored at -20°C or for short periods at 0°C.
- iii. The cell suspension is well-mixed, then 10 λ is added to each well and the tray is vortex-mixed.
- iv. After a 10 minute incubation at room temperature, the cell-serum mixtures are drawn into 10 λ Drummond Microcaps, or capillary tubes of 8 mm outside diameter (OD) x 32 mm length, and sealed with Seal-Ease clay. The tubes are previously boiled and air-dried.
- v. The tubes are spun for $\frac{1}{2}$ to 1 minute at 13,000 g in a Micro-hematocrit centrifuge model MB.
- vi. The tubes are affixed onto a metal plate with double-sided tape and placed at a 45° angle for 1 hour minimum at room temperature, as illustrated in Figure 2.

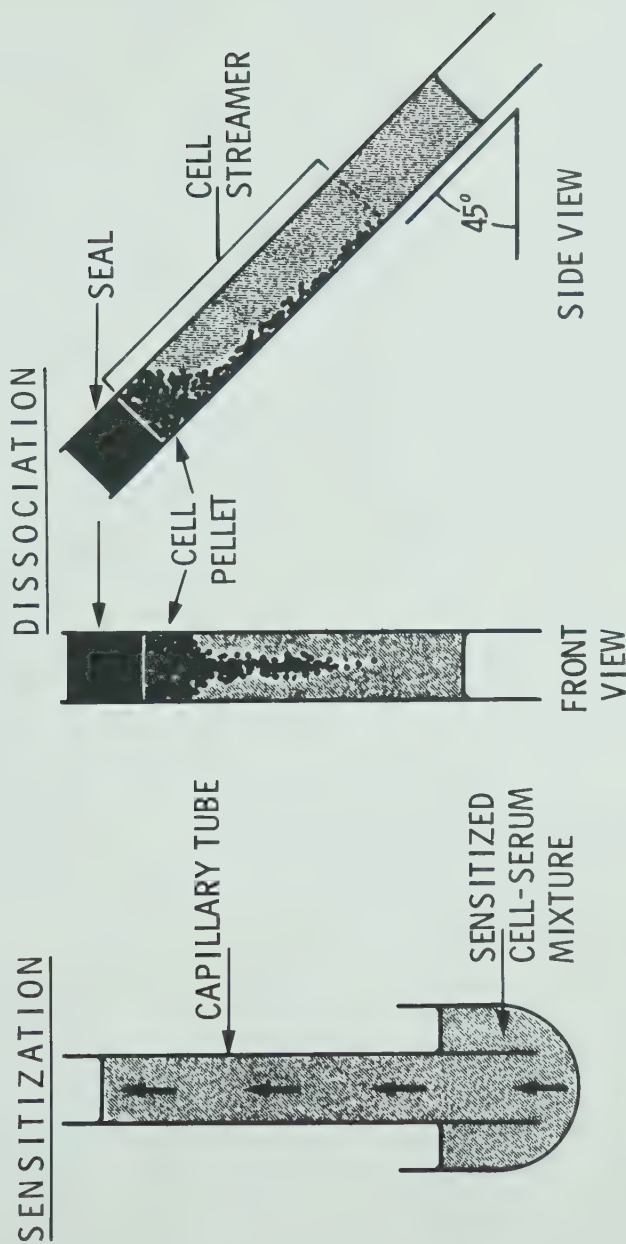
c. Reading the tests

- i. A specially made microscope (with a 1 \times objective and a 10 \times eyepiece) is used to examine the tubes. The tubes are placed in slotted metal holders, alongside a scale in millimetres.
- ii. The length of the cell streamer formed under gravity is measured. The length of this streamer is inversely proportional to the concentration of antibody in the serum. The streamer lengths of the test sera are compared to that of control normal AB serum, which is usually about 20 mm.

$$\frac{\text{length of test streamer}}{\text{length of control streamer}} \times 100 = \text{percent migration}$$

FIGURE 2

SCHEMATIC REPRESENTATION OF THE CAPILLARY AGGLUTINATION TEST (CAT).



Source: Thompson, J.S., Severson, C.D., Coppleson, L.W. and Stokes, G.: Leukocyte capillary agglutination: Demonstration of additional leukocyte antibodies in cytotoxically "monospecific" antisera. In: Histocompatibility Testing 1970. pp. 587-593.

Figure 1, p. 588.

The reading is as follows:

	% migration
1 = negative	80 - 100
4 = doubtful positive	50 - 80
6 = positive	30 - 50
8 = strong positive	0 - 30

d. Solutions used

All solutions are stored at 4°C except the neutralizing solution, which is stored at room temperature.

- i. GEPS: 0.1% neutralized gelatin in 0.3% EDTA; 0.26% Na_2HPO_4 ; 0.85% NaCl, in double distilled water, pH 7.2.
- ii. Shocking solution: 0.25% EDTA; 0.26% NaCl, in double distilled water.
- iii. Neutralizing solution: 9.0% NaCl; 2.5% EDTA; 5.0% Na_2HPO_4 in double distilled water.

e. Using the test

The CAT test was used mainly to detect an unspecified antibody, in the same systems as cytotoxicity and leukoagglutination. Each test was performed in triplicate. It was also used in an attempt to specify an antibody (in comparison to the specificity by cytotoxicity).

There is usually no contamination of the leukocyte preparation with erythrocytes. However, even a high contamination does not detract from the streamer formation. The red cells pack underneath the leukocytes in the capillary tubes, and do not detach until the leukocytes have entered streamer formation. This is confirmed by Thompson (1968), even with antisera against the erythrocytes.

The rate of formation of the cell streamer was studied in some sera. In two series, the migration with normal serum was followed in

8 tubes. The rate of migration was constant at about 0.3 mm per minute, slowing after 45 minutes. The rate of 3 tubes of one series is plotted on Figure 3. Five sera from multiparous women were also studied; three showed this pattern, though slowing down closer to 60 minutes. One serum was strongly positive throughout; another had a short streamer at first but migrated at the same rate and had caught up in length with the other tubes after 2 hours (Figure 4). At 45 or 60 minutes, it would be called a "4". The reading time of 60 minutes appears to be the best; weakly positive sera might "catch up" after this time.

20 μ l capillary tubes and 25 μ l dispensers were used at first, but later 10 μ l tubes were used and the microscope was especially made for this tube size. The average "normal" length of 20 mm is easily readable, as are the variations from 0 - 100%. Thompson et al are now using 1 μ l microcaps, with 1 μ l each of serum and cells, under 2 μ l of oil (1971). In one series using these quantities, the average migration with normal serum was 7 mm, which is readable but only with difficulty with our present microscope.

Thompson and Severson have recently described a modification with methyl cellulose (1972). The blood, with 1 ml of 2% methyl cellulose, is centrifuged to give a buffy coat, which is then treated with shocking solution and the method continued as usual. In using this method for a short period of time, I found it yielded fewer cells, and sometimes caused non-specific clumping and poor streamers.

The CAT test is performed with a leukocyte preparation. One series of sera was tested to determine whether pure lymphocytes would react in the same way. The lymphocytes were isolated by the Ficoll-

FIGURE 3

RATE OF STREAMER FORMATION IN CAPILLARY
AGGLUTINATION WITH NORMAL SERA.

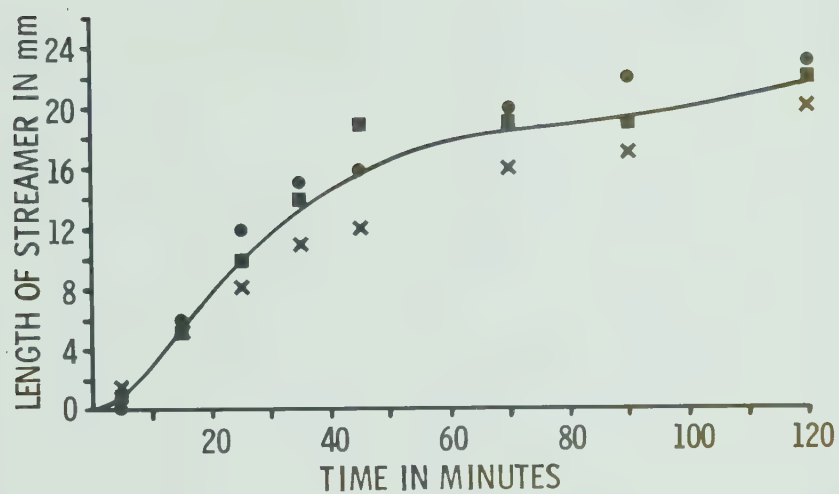
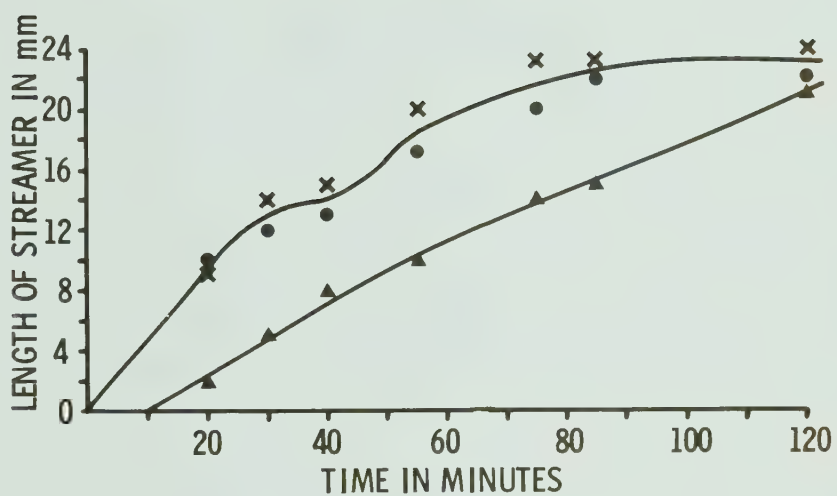


FIGURE 4

RATE OF STREAMER FORMATION IN CAPILLARY
AGGLUTINATION WITH TEST SERA



Isopaque technique (Thorsby, 1969), at 85% purity. 14 sera were chosen for their capillary agglutinating activity. All but four also had cytotoxic activity. None of the sera gave a negative reaction, indicating that a lymphocyte antigen is being detected. Thompson et al (1970) state that the test works equally well with leukocytes or pure lymphocytes.

B. Standardization of Techniques

This will be described under two main headings: "technical" reproducibility, to see if the following interactions are reproducible; a) 5 cell preparations from the same person on the same day tested against a panel of sera, and b) cell preparations from the same person on 5 different occasions tested against a panel of sera, and "biological" consistency of sera obtained on a number of different occasions tested against one cell preparation.

1. Technical Reproducibility (Table 2)

a. 30 or more sera were tested five times by each method with cells from the same person and on the same day.

i. Lymphocytotoxicity (CYT) - 53 sera were tested, of which 26 were negative, 25 were positive, and two were equivocal, with reactions ranging from 1 through to 6. Comparing just the definitely positive or negative sera, there was a 1.2% incidence of false positives in 255 tests, and a 1.2% incidence of false negatives. Thus there is a total error of 2.4%. In 1968, Mittal et al published preliminary data on lymphocytotoxic reproducibility in 12,246 tests. Their rate of error was

TABLE 2

TECHNICAL REPRODUCIBILITY OF THE THREE TECHNIQUES,
USING THE SAME CELL DONOR THROUGHOUT.

Technique	Series	Total Sera	Number of Replications	Total Tests	Total Error	% Error																
CYT*	a¶	51	5	255	6	2.4																
	b§	36	5	180	8	4.4																
CATδ	a	35	5	175	5	2.8																
	b	39	5	195	7	3.6																
AGG†	a	34	4 or 5	166	6	3.6																
	b	24	5	120	12	10.9																
<table><tr><td></td><td></td><td>Negative</td><td>Positive</td></tr><tr><td></td><td>CYT</td><td>1 or 4</td><td>6 or 8</td></tr><tr><td></td><td>CAT</td><td>1 or 4</td><td>6 or 8</td></tr><tr><td></td><td>AGG</td><td>-</td><td>weak to ++++</td></tr></table>									Negative	Positive		CYT	1 or 4	6 or 8		CAT	1 or 4	6 or 8		AGG	-	weak to ++++
		Negative	Positive																			
	CYT	1 or 4	6 or 8																			
	CAT	1 or 4	6 or 8																			
	AGG	-	weak to ++++																			

*CYT - Cytotoxicity

δCAT - Capillary agglutination

†AGG - Leukoagglutination

¶a - 5 cell preparations on the same day, tested against a panel of sera.

§b - Cell preparations on 5 different occasions, tested against a panel of sera.

5.16% in their first series of 1,416 tests. With the alterations in technique of first using multiple syringe dispensers and then wiping the tips of the needles during the addition of cells, their error rate decreased to 3% in 4,503 tests, then to .95% in 6,327 tests. We use the multiple syringe dispensers only for the dispensing of sera, not cells.

Possible reasons for error are many and varied. There may be a transfer of a positive serum to other wells, on the syringe needle, when adding cells or complement. Wiping the tips of needles during addition helps prevent this. Further, there is the possibility of sera "skipping" into another well if cells or complement are added too vigorously. False negatives may be due to improper mixing of sera and cells, or the complement may not be active (to allow recognition of this a positive serum is included on each tray). Improper storage (such as frequent freeze-thawing) could cause loss of activity and thus a false negative.

Visual assessment of percentages of stained cells may lead to error. It has been shown that unequal distribution of cells in a small area of the well may lead to overestimation of dead cells (Mittal et al, 1968). Terasaki and Mickey claim reproducibility also depends on the quality of the serum (1971). Sera with a high percentage of strong reactions give fewer discrepancies. Thus a reason for "doubtful" reacting sera is that weak antisera, killing less than 100% of the cells, vary considerably from day to day and test to test (Singal et al, 1970). All tests were done in triplicate to minimize this possibility of error.

ii. Leukoagglutination (AGG) - 35 sera were tested, of which 17 were negative, 17 were positive, and one was equivocal. There was a 1.2%

incidence of false negatives, and a 2.4% incidence of false positives, in 166 tests, giving a total error of 3.6%.

A possible reason for false positives in this test is the transfer of a positive sera to other tubes by means of the Pasteur pipette. False negatives could be caused by improper mixing of cells and serum, or by an insufficient number of cells in the cell suspension.

iii. Capillary agglutination (CAT) - 38 sera were tested, of which 16 were negative, 19 were positive, and three were equivocal, with reactions ranging from 1 to 8. Omitting these equivocal sera, there was a 1.1% incidence of false positives and 1.7% incidence of false negatives, in 175 tests. The total error was thus 2.8%.

False positive reactions may be caused by the presence of sediment in a serum which has not been centrifuged sufficiently - this will coat the cell button in the capillary tube and prevent cell streaming. There could also be transfer of a positive serum to other wells by the tip of the syringe or during vortexing. If the tubes are left for too long a time at a 45° angle, some weakly positive sera may form a longer streamer which is not conclusively positive.

Failure of cell preparations to give any streamers may be due to platelet contamination, insoluble material in the sera or solutions, a cell preparation that is more than a few hours old, or blood that is not prepared immediately (Severson and Thompson, 1968).

b. A panel of sera was tested with cells from the same individual but obtained from bleedings on five different days.

i. Lymphocytotoxicity (CYT) - 38 sera were tested, of which 16 were negative, 20 were positive, and two were equivocal. There were 4.4%

false negatives in 180 tests (disregarding the equivocal sera) - giving a total of 4.4% error.

Terasaki and Mickey studied discrepancies in reactions with cells taken at two different times (1971). They found an error rate of 2.15% in 35,100 tests. The range for different cells on different trays was 1.34% to 4.68%. They found a wide variation in the quality of the sera - some had no discrepancies, others had more than 5% error. Again, the stronger sera yield more reproducible results.

ii. Leukoagglutination (AGG) - 25 sera were tested, of which 14 were positive, 10 were negative, and one was equivocal. There were 1.7% false positives and 9.2% false negatives in 120 tests - a total of 10.9% error.

iii. Capillary agglutination (CAT) - 45 sera were tested, of which 25 were negative, 14 were positive, and six were equivocal. There were 0.5% false positives and 3.1% false negatives in 195 tests - a 3.6% total error.

2. Biological Consistency

A panel of sera, among which were several taken at different time intervals from six different patients, was tested with cells from only one bleeding. This was performed two different times with leukoagglutination (AGG) and five times by capillary agglutination (CAT) and cytotoxicity (CYT). The positive controls were consistently positive; the negative controls were always negative. One patient was negative by all methods for all samples, another was consistently positive. The results from the other four patients are shown on Table 3. Only one patient (IV) showed the same reactivity by all three methods. The others

TABLE 3

BIOLOGICAL CONSISTENCY OF SERA FROM FOUR PATIENTS ON DIALYSIS

Patient	Test	Serum Sample															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
I	CYT	+	+	-	-	-	+		+	+	+	+	+	+	+		
	CAT	+	-	-	-	+	+		+	+	+	+	+	+	+		
	AGG	-	-	-	-	-	-		+	+	-	-	-	-	-		
II	CYT	-	-	-	-	+	+	+	+	+	+	+	+	+			
	CAT	-	+	-		-	+	+	+	+	+	+	+	+			
	AGG	-	-	-	-	-	-	-	-	-	+	+	-	+			
III	CYT						-	-	-	-	+	+	+	+	+	+	+
	CAT	-	-	-	-	-	+	-	-	-	-	+	+	+	+	+	+
	AGG						-	-	-	+	+	+	+	+	+	+	+
IV	CYT	-	-	-	-	+	-	-	+	+	+						
	CAT	-	-	-	-	+	-	-	+	+	+						
	AGG	-	-	-	-	+	-	-	+	+	+						

+ indicates a positive reaction

- indicates a negative reaction

showed great variability in which tests were positive with each serum sample.

3. Comparison of Reproducibility

There is no difference between the technical reproducibility of CYT or CAT. This 2.4 to 4.4% is an acceptable rate of error, and it shows that cells, at least of healthy subjects, do not change with time. However, it has been shown that cells of critically ill persons have cells varying in reactivity between testings (Terasaki and Mickey, 1971). Agglutination had a higher percentage of error when cells from different times were tested (10.9%). Partly for this reason, it was discontinued during the study.

In the biological consistency of sera, it can be seen that the sera of these dialysis and transplant patients are ever-changing in reactivity, because of their transfusions, transplants and immunosuppression. It appears that antibodies detected by the different methods change at different rates in the same patient, and different rates in different patients. This, then, needs further study. On the basis of the technical reproducibility, there is no reason to doubt that these reactions represent true biological changes in reactivity. Those patients who are either negative or positive over a long period of time rarely have false reactions. This has been borne out in the kidney patient screens. With one patient who has never received a transfusion or transplant, there is much less than 1% false positive reactions with his sera.

A series of 38 sera from "normal" individuals, obtained from the medical examinations of new University of Alberta employees, was tested by the three methods against five cell types. All of the tests performed

were negative.

C. Sources of Materials

I. Sera

- a. Sera from parous women - These were obtained at delivery by the Obstetrical Service at the University of Alberta Hospital, and any follow-up samples from the woman and/or her husband were obtained either at their home or at the laboratory.
- b. Sera from postmenopausal multipara - Pre-transfusion sera of the postmenopausal multipara were obtained from the Blood Bank of the University of Alberta Hospital, as were those obtained immediately after transfusion. Subsequent blood samples from the woman and her husband were taken at their home, at the laboratory, or with the co-operation of their family doctor if they lived out of town.
- c. Sera from dialysis patients - These were obtained monthly from the Dialysis Unit or the Blood Bank of the University of Alberta Hospital. It should be noted that since these patients have a high incidence of Australia antigen, special precautions were taken in handling their sera (Sutnick et al, 1971).
- d. Sera samples from transplanted patients - These were obtained from the Out Patient Department of the University of Alberta Hospital.
- e. Negative control sera - This was serum from an AB blood donor with no known exposure to HL-A antigens.
- f. Positive control serum - This was pooled from renal patients with multispecific antibodies.
- g. Storage of sera - The sera were stored at 0°C before testing. The remainder of the sera was stored at -20°C or -60°C and thawed before

use in subsequent tests. Any serum used several times at intervals was stored in 0.2 to 0.4 ml quantities, so that there was usually only one thawing of each sample tested.

h. Complement - Rabbit serum was used as the source of complement in lymphocytotoxicity. It was either pooled from several rabbits and stored at -60°C until use, or lyophilized complement was sometimes employed.

2. Cell Preparations

a. Cells from husbands of multipara - Blood samples for cell preparations on husbands of postmenopausal multipara and postpartum multipara were obtained at the same time as serum samples were taken from the women.

b. "Cell panel" for screening - Cells from five volunteers were used to test each serum for antibodies by each technique. This cell panel was at first random, but became a constant panel representing 17 antigens (all HL-A antigens but 13, 27 and 28, and all the W antigens but 15, 16, 18, 21 and 29).

CHAPTER IV

RESULTS

A. Use of Capillary Agglutination to Detect HL-A Sensitization

I. Anamnestic Responsea. Introduction

This research set out to establish the possible clinical importance of latent sensitization to HL-A antigens. Hyperacute rejection may occur with a negative crossmatch, as previously discussed. It is of importance to determine whether these cases are non-antibody reactions, or whether the antibody is in a "latent" form, undetectable by present methods, prior to re-exposure to histocompatibility antigens.

Antibodies can disappear from the circulation either as a result of intense immunosuppressive treatment or with the passage of time. Latency with immunosuppression has been described earlier, as has the loss of antibody with time among dialysis patients. Antibodies in multiparous women are also lost with time. Thirty to fifty percent of multipara develop leukocyte antibodies to incompatible antigens of their husbands. These antibodies do not persist indefinitely, but their specificity narrows with time (Payne, 1962) and they disappear after several months or years (Cohen and Sackett, 1971). With their disappearance, however, immunologic memory may remain. In such a case, there would probably be a negative pre-transplant crossmatch with lymphocytes, but an allograft or transfusion which shared the husband's antigens could cause rapid reappearance of the antibody. With a graft, acute humoral rejection might occur. This possibility could explain instances of

acute rejection in some multiparous recipients (Starzl et al, 1968; Stewart et al, 1970).

The aim, then, was to see if blood transfusions will reveal latent HL-A sensitization in multiparous women several years after their last pregnancy. Lymphocytotoxicity (CYT), capillary agglutination (CAT), and leukoagglutination (AGG) were all used, in case one of the last two methods could detect antibody in its latent state where cytotoxicity cannot. This might occur if the antibodies belong to other classes of antibodies than cytotoxic ones, or one of the other tests may be a more sensitive technique.

b. Preliminary study

Multiparous and nulliparous women were selected from the Blood Bank files, if they were between the ages of 45 and 65 (thus several years since their last pregnancy and classified as postmenopausal) and had received a blood transfusion of two or more units one to three years before the study was begun. The doctors of these women were then contacted and permission was requested to continue the study. Blood was then taken from all women who agreed to participate, and their serum was tested for antibody by CYT and AGG. The serum of any woman with antibodies was then tested against her husband's cells by all three methods, and the husband's HL-A profile was determined. A total of 40 multiparous women were tested, with the average number of pregnancies being five, and eight nulliparous women were tested.

Among the eight nulliparous women, none had antibodies by any test. There were 10/40, or 25%, with antibodies in the multiparous group (Table 4). These antibodies, then, were still detectable two to

TABLE 4

DETECTION OF ANTIBODIES IN POST-TRANSFUSION
POSTMENOPAUSAL WOMEN - PRELIMINARY STUDY

	Average Units of Blood Received	Average Age	Number with Antibodies 1-3 years Post-transfusion
Postmenopausal Multipara	4	52	10/40 (25%)
Postmenopausal Nullipara	5	55	0/8 (0%)

three years post-transfusion.

Of the six women tested with their husband's cells, five were positive by at least one of the three tests (Table 5). The sixth woman had had at least three transfusions prior to those considered here. The antigen profiles of the husbands, and possible antibodies formed by the women are also shown on Table 5. It can be seen that the five women positive to their husband's cells developed antibody to at least one of the husband's antigens. Of the 10 women with antibody, six were anti-HL-A2.

This study was then extended, since it was found that postmenopausal multiparous women after blood transfusion do possess antibodies. In addition, as shown on Table 5, CYT, CAT and AGG reactions did not correspond, as three of the sera reacted by only one of the methods each.

c. Main study

The test group consisted of multiparous women several years after their last pregnancy; controls were men and nulliparous women. Only women from the gynecological ward were considered, to eliminate a possible association with immunological disease, and to minimize the possibility of these women having had previous transfusions. The men in the test group were from various wards. There were 20 patients in each of the groups tested, and the average number of units of blood transfused in each group was four, ranging from one unit (450 ml) to 20 units. The diseases from which the patients suffered were non-immunological, being mainly uterine fibroids or uterine prolapse among the women and heart surgery among the men. The average number of pregnancies in the test

TABLE 5

REACTIVITY OF POST-TRANSFUSION ANTISERA - PRELIMINARY STUDY

Gravida	Units of Blood Rec'd	HL-A Antigens of Husband	Reactions with Husband's Cells			Probable ^{a,b} Antibody
			CYT	CAT	AGG	
4	4	HL-A 2,3,7	-	+	-	<u>HL-A7</u>
3	4	HL-A 2,8,12	+	+	-	<u>HL-A2,7</u>
5	2	HL-A2,7,10,Te60	+	-	-	<u>HL-A2</u>
4	8	HL-A2,7,11	-	+	-	<u>HL-A2</u>
7	2	HL-A7,±9,10,Te60	+	+	+	<u>HL-A7</u>
3	2 (3 units prior to these)	HL-A3,5,9	-	-	-	<u>HL-A2</u>
5	2					HL-A9...
7	3					
6	2					HL-A2...
3	2					HL-A2

^a - Specificities underlined are against husband's antigens

^b ... - Additional uncharacterized antibodies

group was four, each woman having had at least two pregnancies.

Serum samples were obtained pre-transfusion, and five days, three weeks and six weeks post-transfusion. All of the serum samples were tested in the usual way, by all three methods. Any positive sera were tested against the husband's cells, which were typed for HL-A antigens. The antibodies were further characterized by their reactions against a panel of 50 or more cells of known HL-A profile, by lymphocytotoxicity only.

There was no antibody detected to the cell panel prior to the surgery and blood transfusion (Table 6). Following this, antibodies appeared in 8 of 20, or 40%, of the multiparous group. This is significantly greater ($p < 0.05$) than the 1/20, or 5%, of the control group. It seems evident that antibodies have developed in those in whom there was previous exposure to HL-A antigens.

If this is latent sensitization, the antibodies should be mainly against the husband's incompatible antigens. Of the seven women tested against the husband's cells, six are positive by all three tests. The seventh woman had had a child by a first husband. Table 7 shows that all six women developed antibody against at least one of the husband's antigens. The most likely explanation for additional antibodies formed is antibody cross-reactions, discussed previously. Thorsby et al (1970) describe HL-A5 and Te50 (4C*) as one of the antigen groups against which cross-reacting antibodies are often produced. This would explain the first and third cases on Table 7.

Four of the women developed antibody by five days post-transfusion, indicating pre-sensitization in at least these cases.

TABLE 6

DETECTION OF ANTIBODIES BEFORE AND AFTER TRANSFUSION

	Average Age	Sera Reacting Against Cell Panel	
		Pre-transfusion	Post-transfusion
Controls (20)	59	0	1 (5%)
Postmenopausal Multipara (20)	40	0	8 (40%)

TABLE 7
REACTIVITY OF POST-TRANSFUSION ANTISERA

Gravida	Number of Transfusions at time of Hysterectomy	HL-A Antigens of Husband	Reaction with Husband's Cells	Probable ^{a,b} Antibody	Comments
4	2	HL-A 2, 5, 9, Te60	positive	anti <u>HL-A 5</u>	Later developed antibody to Te50.
4	4	HL-A 3, 9, 12, W15	positive	anti <u>HL-A 9, 12...</u>	Became multispecific. Transfusion in 1964.
5	2	HL-A 3, 7, 10, Te50	positive	anti <u>HL-A 3, 5</u>	Later developed anti-body against the <u>Te50</u> series. Transfusion in 1954.
5	5	HL-A 1, 3, 17	positive	anti <u>HL-A 1, 2...</u>	
2	2	HL-A 2, 8, 9, Te60	positive	anti <u>HL-A 2, 7, 9</u>	Became multispecific.
3	2	HL-A 2, W32, Te60	positive	multispecific	
4	2	HL-A 3, 7, Te50	negative	anti HL-A 2, 9	
12	1			anti HL-A 3, 5, 9, 12	Had a child by a previous husband.

^aThe specificities underlined are against husband's antigens

^b... = additional uncharacterized antibodies

In comparing results of the three tests, there are some discrepancies. In six instances, leukoagglutination (AGG) failed to detect antibody when the other tests did. In no instance was AGG positive if the others were negative. There were three cases where capillary agglutination (CAT) detected antibody before transfusion. In one of these three, an antibody positive against her husband's cells appeared strongly by all three tests. However, as her pre-transfusion antibody was not directed against her husband's antigens, the significance of the positive CAT pre-operatively is not clear. In the other patients, a man and a multiparous woman, though the CAT test was positive pre-operatively, no cytotoxicity to lymphocytes developed post-operatively.

Another observation is that of the four women who were followed to the disappearance of their cytotoxic antibody, two remained positive by CAT and AGG.

2. Antibody Screening of Sera from Dialysis and Transplant Patients

a. Patients on hemodialysis

Monthly screening against the cell panel was performed for 22 months on the sera from 27 uremic patients, using lymphocytotoxicity (CYT) and capillary agglutination (CAT). Leukoagglutination (AGG) was also used for 16 of these months.

Terasaki et al (1971) reported 20 to 25% of dialysis patients to have cytotoxins at any one time, with a maximum of 60% sensitization when the loss of antibody with time is considered. Our results agree with this, though our percent of cytotoxic antibodies at any one time is higher (average of 35%). The percent with CAT and AGG antibodies is the same at any one time, but with agglutination there is only a 46% total

sensitization. One-third of our patients (9/27) were humorally unresponsive after one year and 10 transfusions.

In analysing the results of CYT, CAT, and AGG for each responsive patient, these patterns emerged:

- i. One-half formed antibodies of all types, against certain cells of the panel and at approximately the same time. In some cases either method detected antibodies against other cells. Figure 5 shows the reaction pattern of one of these patients.
- ii. One-quarter developed mainly cytotoxic antibodies, as illustrated by the patient's reactions on Figure 6. This patient had no leukocagglutinating antibodies.
- iii. One-quarter formed antibodies that were mainly CAT-reactive, though there may be correlations within some of the cell types. Figure 7 shows the reactions of one of these patients. This particular patient had a specific antibody to HL-A2. The CAT antibody appears to be of much wider specificity.

257 sera were tested by these methods over a period of seven months. Figure 8 shows the CYT, CAT, and AGG reactions of the 132 positively reacting sera. 62 of the antisera were reactive by all three methods, but over one-half were positive by only one or two of the three methods. The number of sera in each classification are in patient-months, the patients being represented in an unequal fashion each month. Over this time period, five patients either received kidney transplants or died, and seven new patients were added to the program. There is no significant difference between the percentages in each classification, as obtained at the beginning and end of the time period, so no bias was added in this respect.

b. Transplant patients

The sera of all transplant patients were screened for antibody

FIGURE 5

REACTION PATTERN OF A DIALYSIS PATIENT WITH CYTOTOXIC, CAPILLARY AGGLUTINATING AND LEUKOAGGLUTINATING ANTIBODIES.

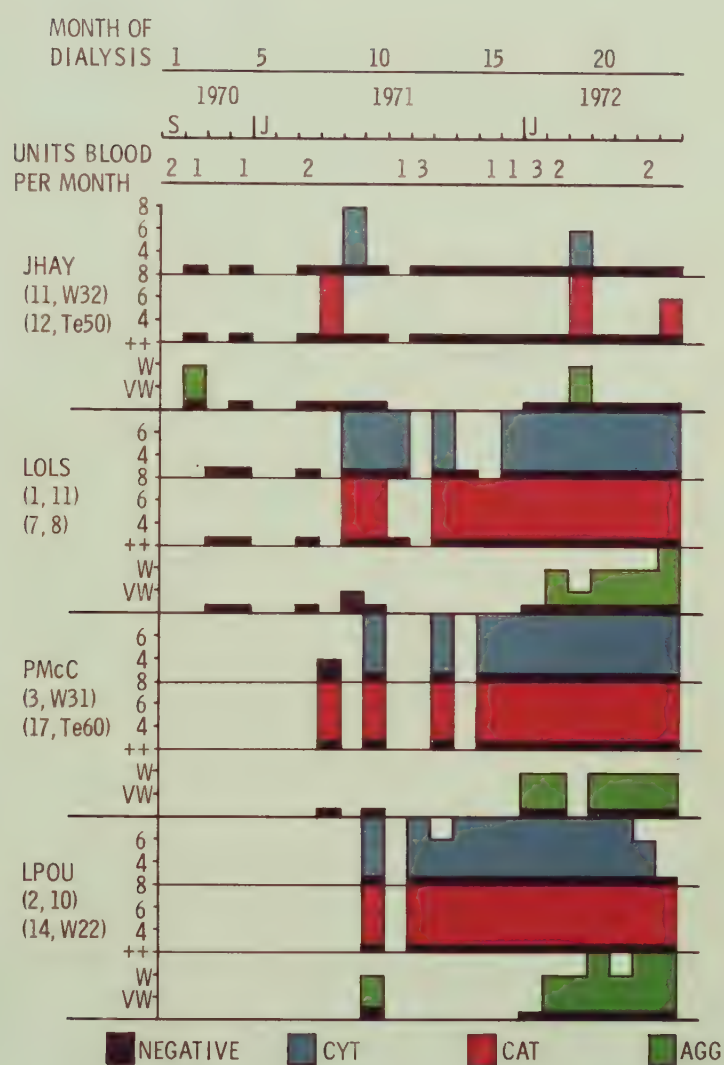


FIGURE 6
REACTION PATTERN OF A DIALYSIS PATIENT
WITH MAINLY CYTOTOXIC ANTIBODIES.

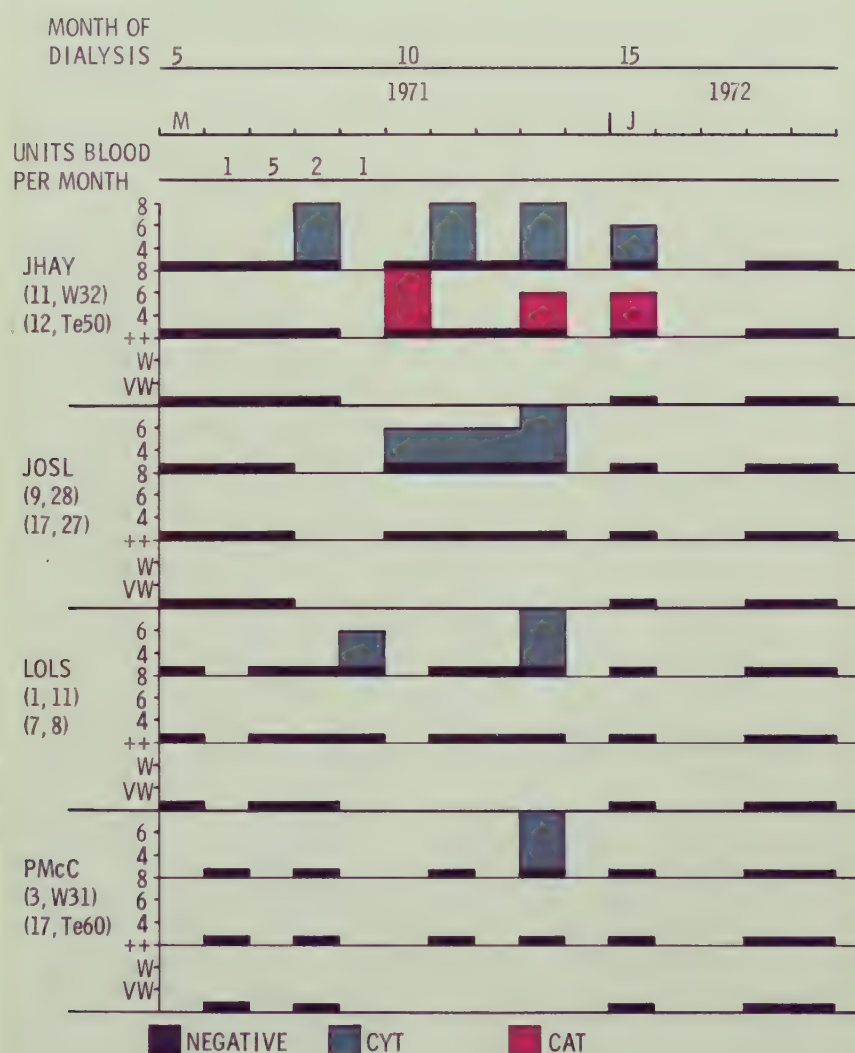


FIGURE 7

REACTION PATTERN OF A DIALYSIS PATIENT WITH MAINLY
CAPILLARY AGGLUTINATING ANTIBODIES.

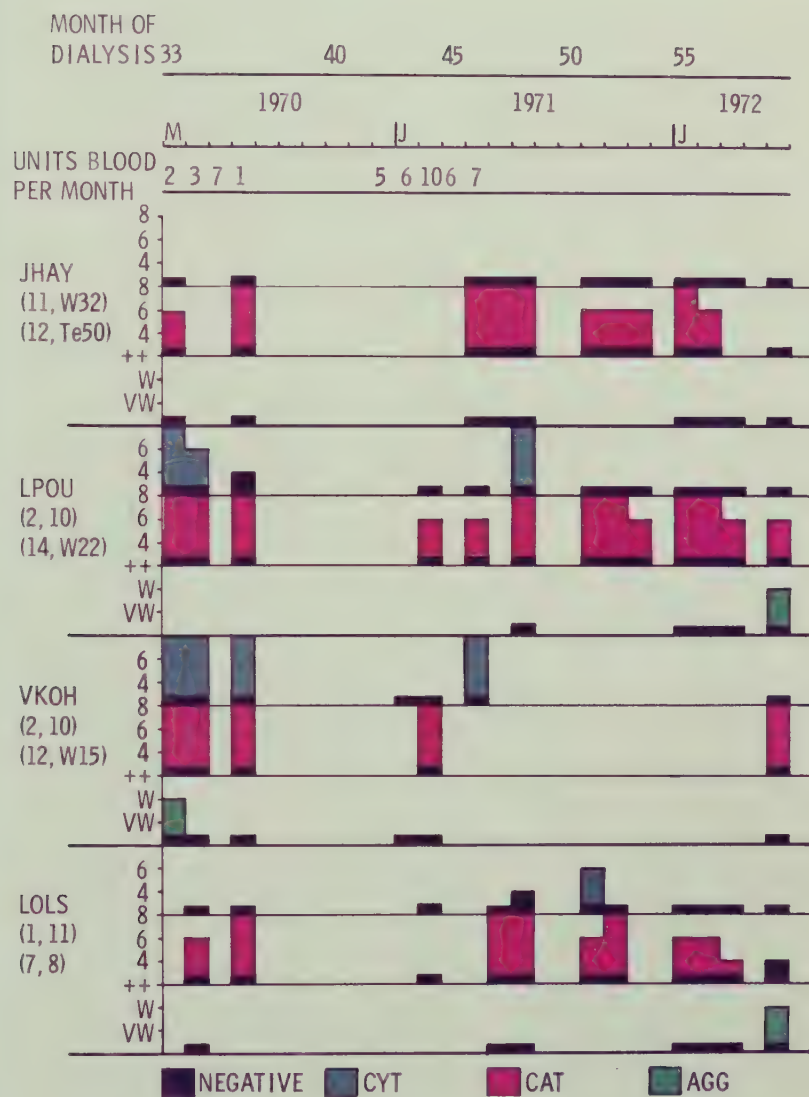
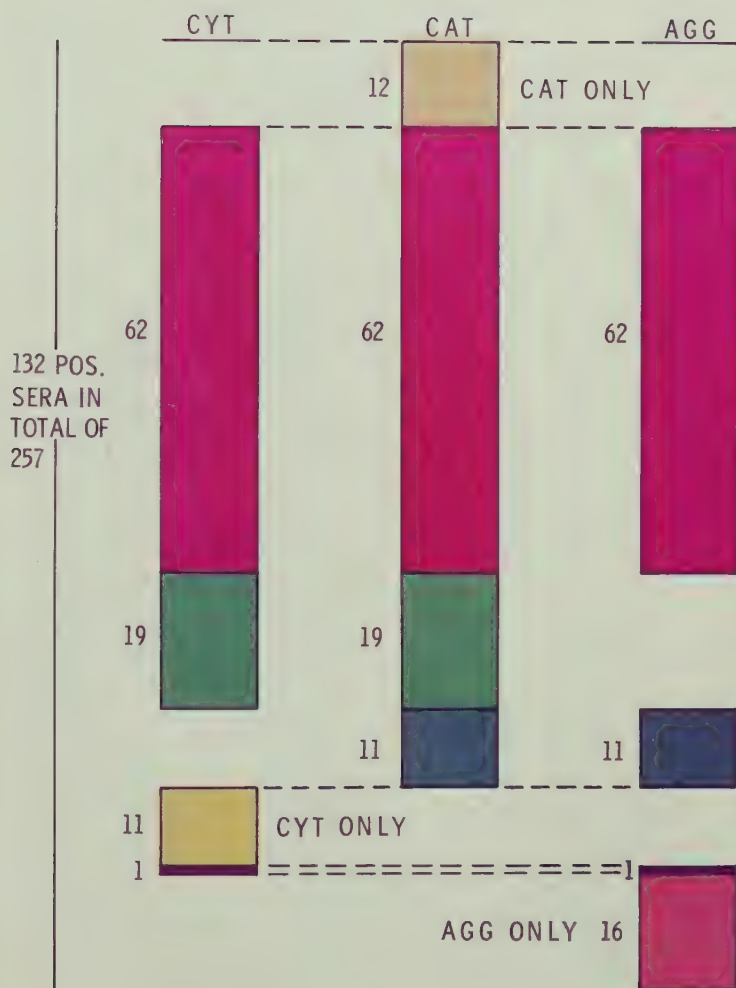


FIGURE 8

CYT, CAT, AGG PATTERNS OF 132 ANTISERA FROM PATIENTS ON DIALYSIS.



as frequently as possible, this depending on how often they came to the hospital after the transplant. In addition, most patients were tested monthly while on dialysis before transplant.

The CYT and CAT screening results of the last pre-transplant sera of 14 patients were studied in relation to subsequent rejection episodes. This was not the crossmatch, which was not performed for CAT and was negative in all cases by CYT against the widest spectrum serum as well as a recent serum. The clinical evaluation of rejection in each transplant was graded by three clinicians independently - they did not know the antibody reactions of a given recipient pre-transplant serum. Table 8 shows the results of this retrospective analysis, and indicates a possible "beneficial" effect of CAT antibody, for there is a significant association between the presence of CAT antibody and the absence of major acute rejection in the first three months after transplantation ($p < 0.05$). Attempts were made to correlate early rejection with other factors. Neither the presence of cytotoxic antibody (Table 9), the total blood transfused pre-transplant, or the rate of blood transfusion showed a significant correlation. There was no real difference in the degree of HL-A mismatch between donor and recipient in the two CAT groups. In the CAT positive group, all kidney donors had two to three HL-A antigen mismatches with the recipients (mean = 2.4) and in the CAT negative group there were one to four antigen differences between donor and recipient (mean = 2.5).

Among 15 patients tested for several months after transplant, the total antibody frequency was 40%. At any one time there was about 15% antibody. Of the six patients with antibody, five show correlation

TABLE 8

STATISTICAL INVERSE CORRELATION BETWEEN CAPILLARY
AGGLUTINATION AND EARLY REJECTION

Capillary Agglutination	12 Weeks Post-Transplant	
	No Rejection	Acute Rejection
Positive	5	0
Negative	2	7

$$\chi^2 = 4.9776$$

$$p = <0.05$$

TABLE 9

LACK OF CORRELATION BETWEEN LYMPHOCYTOTOXICITY
AND EARLY REJECTION

Lymphocytotoxicity	12 Weeks Post-Transplant	
	No Rejection	Acute Rejection
Positive	2	1
Negative	5	6

Not significant

between CYT and CAT, and one had mainly CAT antibodies. In no patients without antibodies before transplant was there antibody detected immediately after transplant. In only one woman who had antibody before transplant the antibody has remained for a long period of time. In others (five patients) antibody of both types eventually disappeared. In several patients there were CAT or CYT antibodies on only one occasion - these were considered negative.

3. Antibody Screening of Sera from Parous Women

a. Sera taken at delivery from parous women were screened for antibody by all three methods, capillary agglutination (CAT), lymphocytotoxicity (CYT), and leukoagglutination (AGG). The percentages of positive sera have been grouped according to number of pregnancies (Table 10). Though it may appear that in general AGG is more sensitive than CYT, and CAT more than AGG, there is no indication here of whether the same sera are positive by each method or whether each method detects a different group of antisera. To answer this, an analysis was made of 253 parous sera tested against a panel of cells which was constant within a serum for each of the CYT, CAT, and AGG tests. Of these, 86 reacted positively by at least one of the methods as shown in Figure 9. These sera were grouped on the basis of which tests they were reactive with (Figure 10). As in the similar analysis of sera from dialysis patients, some sera reacted by all three methods but over one-half were reactive by only one or two of the three tests.

Greater numbers of parous sera have been screened by cytotoxicity alone, and by two of the three methods (CYT and AGG). Results from these more extensive studies and those of other investigators are found in

TABLE 10
PERCENTAGES OF CYT, CAT AND AGG ANTIBODY IN
SERA FROM PAROUS WOMEN

Gravida	Percent with CYT Antibody	Percent with AGG Antibody	Percent with CAT Antibody
1 & 2	$9/87 = 10.3$	$12/87 = 13.8$	$16/87 = 18.4$
3	$27/127 = 21.3$	$29/127 = 22.8$	$38/127 = 29.9$
4	$19/70 = 27.1$	$20/70 = 28.6$	$24/70 = 34.3$
5	$11/43 = 25.6$	$9/43 = 20.9$	$12/43 = 27.9$
6 or more	$9/39 = 23.1$	$10/39 = 25.6$	$14/39 = 35.9$

FIGURE 9
REACTIVITY OF 253 SERA FROM PAROUS WOMEN.

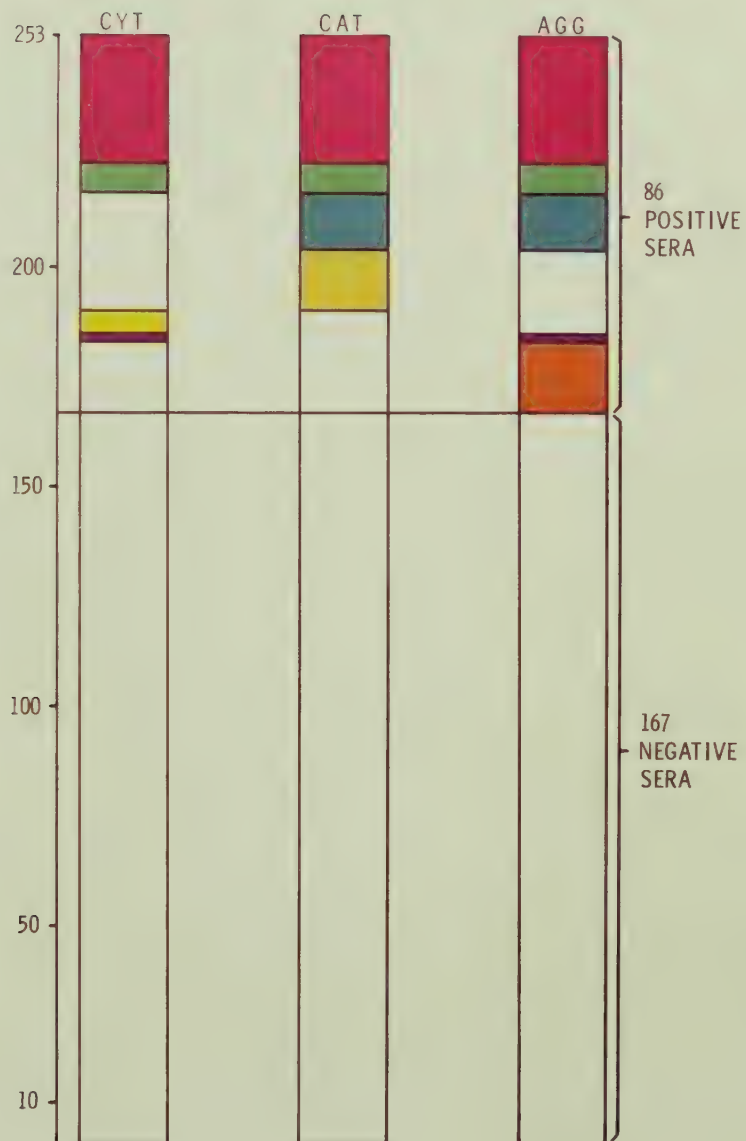
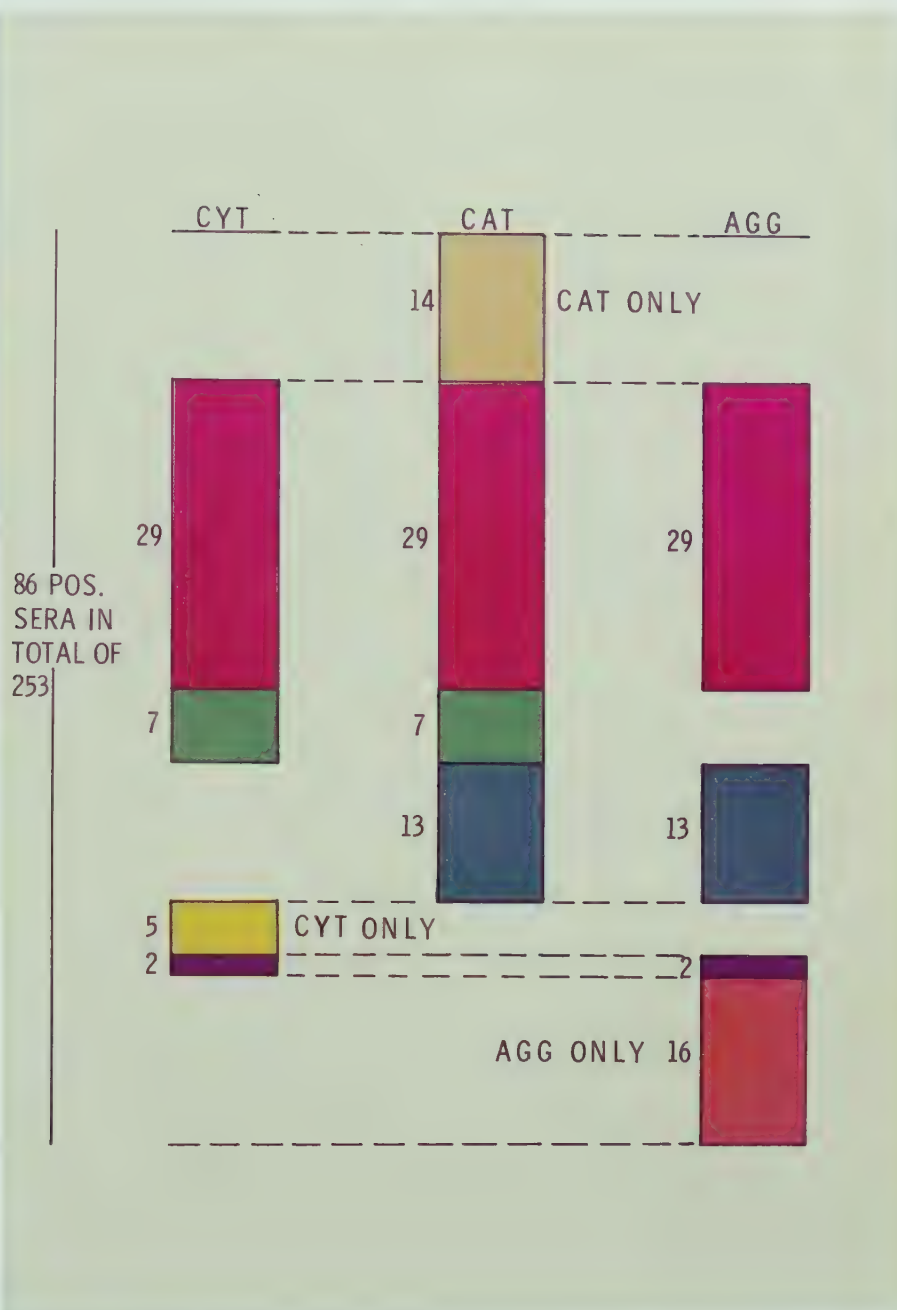


FIGURE 10

CYT, CAT, AGG PATTERNS OF 86 ANTISERA FROM PAROUS WOMEN.



Appendix B. CAT and CYT together have also been used to test a further series of sera. These are analysed in the section on specificity.

b. Ahrons (1971) found a very high percentage of primipara to have antibody at delivery (22% by CYT, 30% by AGG) with an increase to 30% and 34% respectively at four weeks postpartum. Our percentages were much lower (14% by CYT and 19% by AGG). In a preliminary follow-up of 20 primigravida after delivery, none were positive at delivery, 1/20 (5%) developed CYT and CAT antibodies and 3/20 (15%) had AGG antibody at six weeks.

B. Further Analysis of Capillary Agglutination (CAT)

1. Sensitivity

a. Postpartum antisera from parous women were tested in serial dilution with the husbands' cells by CYT and CAT (Table 11). In 15 instances, there were several testings, with sera of different time intervals. In a total of 56 combinations, 14 were positive to the same dilution in both, 18 were positive to a higher dilution by CAT, and 24 were positive to a higher dilution by CYT.

b. Sera from dialysis patients were tested in serial dilution against one or more cell types by CYT and CAT as also shown in Table 11. In some cases, different sera from the same patient were tested. In a total of 19 test combinations, 6 were positive to the same dilution in both, 6 were positive to a higher dilution by CAT, and 7 were positive to a higher dilution by CYT.

c. All three tests (CAT, CYT and AGG) were used in a few cases to compare sensitivity (Table 12). Each detected antibody at a higher titre in some sera than did the two other tests.

TABLE II

SENSITIVITY OF CYT AND CAT IN 56 POSTPARTUM SERA AND
19 SERA FROM PATIENTS ON DIALYSIS

Test Detecting the Highest Titre	Number of sera	
	Postpartum	Dialysis Patients
CYT and CAT	14	6
CYT higher by 1 dilution	11	3
2	8	2
3	4	2
4	1	
	24	7
CAT higher by 1 dilution	8	3
2	8	0
3	2	1
4		2
	18	6
TOTAL TESTS	59	19

TABLE 12

SENSITIVITY OF CYT, CAT AND AGG IN NINE SERA

Test Detecting the Highest Titre	Number of Sera
CAT	1
CYT	2
AGG	0
CYT and CAT	2
CYT and AGG	1
CAT and AGG	2
CYT, CAT and AGG	1

2. Relative Serum Changes with Time

a. In the Anamnestic study, it was seen that two of four women who lost their cytotoxic antibody after transfusion kept their CAT and AGG antibodies, when tested nine months later. Of the eight women who had developed antibody, two lost their AGG antibody first. In all cases, antibody appeared by all three methods at the same time.

b. Of 37 women tested for antibody at delivery and then six weeks postpartum, 15 were negative both times. Six showed changes in presence of antibody (Table 13), and 16 were positive both times. There were 11 women whose titre of antibody changed with time, and 5 whose titre remained the same. The antibodies do not change predictably with time. However the proportion of the different patterns cannot be interpreted as this was a non-randomly selected group, chosen for the reactions of the pre-delivery serum with the cell panel used in the screening.

c. Table 14 shows an example of CAT reactivity lasting a longer time than CYT. The patient received many transfusions during hospital dialysis to June 1970. During five months of intensive home dialysis his anemia improved and no transfusions were required. He then developed septicemia with recurrent anemia, and thereafter had to be given several units of blood. In April 1971 he was given a splenectomy and no longer required blood transfusions.

Table 14 shows the reactions against a panel of cells possessing the HL-A2 antigen, to which he had developed a specific cytotoxic antibody. During his transfusions, his cytotoxic antibody was becoming weaker, reacting against fewer of the seven cell donors used. He then lost his antibody after the five month break. It reappeared to some of the

TABLE 13

CHANGES IN PRESENCE AND TITRE OF ANTIBODY IN 23 WOMEN,
FROM PRE-DELIVERY TO SIX WEEKS POSTPARTUM

Number of Women Exhibiting Each Pattern	Pre-delivery Serum		Serum Six Weeks* Postpartum	
	<u>CYT</u>	<u>CAT</u>	<u>CYT</u>	<u>CAT</u>
1	+	+	-	+
1	-	+	+	+
2	-	-	-	+
2	+	+	-	-
			All Positive (Change in Titre as Indicated)	
5	+	+	CYT↑	CAT↑
1	+	+	CYT↑	CAT=
2	+	+	CYT↑	CAT↓
2	+	+	CYT=	CAT↑
1	+	+	CYT=	CAT↓
5	+	+	CYT=	CAT=

- *
 + antibody present
 - antibody not present
 ↑ increase in titre
 ↓ decrease in titre

TABLE 14

CHANGE IN ANTIBODY WITH TIME, IN THE CASE OF ONE PATIENT ON DIALYSIS

MONTH	J	F	M	A	M	J	J	A	S	O	N	D
<u>Year - 1970</u>												
Units of blood required	Prior 42 → -	-	2	3	7	1	-	-	-	-	-	5
Number of HL-A2 cells positive each month												
CYT			7/7	5/7	2/7	5/7						
CAT			3/3	4/4	4/4	3/3						
<u>Year - 1971</u>												
Units of blood required					Splenectomy 7 → -	-	-	-	-	-	-	-
Number of HL-A2 cells positive each month												
CYT	6	10	6	1/5	3/5	2/2			0/3	0/3	0/3	
CAT	0/7	0/7		3/3	3/3	2/2			2/2	2/2	2/2	
<u>Year - 1972</u>												
Units of blood required	-	-	-	-	-	-						
Number of HL-A2 cells positive each month												
CYT	0/1	0/1	0/1	0/2	0/1	0/1						
CAT	1/1	1/1	1/2	2/2	0/1	1/1						

panel, then was lost again when his blood requirement ceased again. Capillary agglutination was tested against fewer of the panel cells, but remained positive throughout. The sera reactions of this patient have been illustrated earlier (Figure 7), against the regular cell panel, where it can be seen that his sera were reactive by CAT with a wider range of cells than those possessing HL-A2.

d. In the dialysis patient screens, CAT was compared to CYT and AGG with respect to appearance and loss of antibody (Table 15). There is again no predictable change of antibody with time.

3. Specificity

In the CAT test as used in sera screening, we suspected that a non-cytotoxic antibody was being detected (and thus perhaps not HL-A).

Points leading to this belief were:

- i. Among the renal patients, there were 1/4 who formed mainly CAT antibodies and 1/4 who formed mainly CYT antibodies. In analysing sera reactions by patient-months (Figure 8), all three methods overlapped but some sera were reactive by one method and not the other two. The same is true in the analysis of sera from parous women (Figure 10).
- ii. Sera of some of the renal patients reacted with a greater range of cell types in the CAT than CYT test, or vice-versa (Figures 6 and 7). The same was observed with sera from parous women.
- iii. Neither the sensitivity of CYT, CAT, and AGG nor the relative serum changes with time showed a predictable pattern. Each test detected antibody at a higher dilution in some sera, and each detected antibody for differing lengths of time in different sera.
- iv. Thompson et al have cited evidence of CAT-positive sera which do not follow the HL-A inheritance pattern within families

TABLE 15

THE ORDER OF APPEARANCE AND DISAPPEARANCE OF CYT, CAT, AND AGG ANTIBODIES IN DIALYSIS PATIENTS.

Order of Appearance 1 2 3	Number with this Pattern	Order of Disappearance 1 2 3	Number with this Pattern	Number with Anti- body Remaining
CYT CAT AGG	4	CYT CAT AGG	2	9
CYT CAT AGG	2	CAT CYT AGG	1	
CAT CYT AGG	2	CYT CAT AGG	1	
CAT CYT AGG	1	AGG only	1	
CYT CAT AGG	2	AGG CYT CAT	1	
AGG CAT CYT	1	AGG CYT CAT	1	
AGG CYT CAT	1			
Pattern Unknown	3			
TOTALS	16		7	9

(1971). Furthermore, Thompson found that there are two groups of multiparous sera: those where CAT correlates with CYT and those where it does not (personal communication).

Further investigation of the question of CAT specificity was undertaken. In some analyses only CYT and CAT were compared; in others, the AGG test was also employed.

a. Correlation with HL-A

i. A panel of 14 sera, mono- or oligospecific for known HL-A specificities, was tested against a series of 10 cells of known HL-A profile, by both CYT and CAT (Table 16). The ratio of both tests positive: CYT positive: CAT positive was approximately 2:1:1. Some reactions can be explained on the basis of cross-reactivity, for example, two sera mono-specific for HL-A2 reacted by CAT with some cells possessing an HL-A28, known to be cross-reactive with HL-A2; but this cannot explain all of the discrepancies seen here.

ii. The range of CYT and CAT reactivity was examined in the sera of parous women tested against the same five-cell panel by each test. Of the 436 sera examined; 295 were non-reactive, 37 had CAT reactions only, 26 had CYT reactions only, two had CYT reactions on some cells and CAT on others. Only 76 sera had reactivity which included CYT and CAT reactions on the same cell of the panel.

Among these 76 sera with both CYT and CAT antibodies, 21 reacted the same by CYT and CAT against the five cell panel, 39 had CAT and CYT activity with some cells and extra CAT reactions with other cells, nine had extra CYT reactions on some cells, and seven had CAT extra on some cells and CYT extra on other cells (Table 17).

From this analysis, it appears that CAT "dominance" (sera having

TABLE 16
CYT AND CAT REACTIONS OF A PANEL OF 14 SERA TESTED WITH 10 CELL TYPES

HL-A Profile of Cells Tested	HL-A Specificity of Each Antiserum Tested*														
	1,8	2	2	2,28	2,28 17	12+	5	5	12	12, Te60	10, 12	10, 12	7 Te60	13	27
3,11,7,12	-	-	-	-	-	+	-	+	-	+	+	+	-	-	+
2,7,27	-	+	+	-	+	-	+	+	-	-	-	-	+	-	+
1,11,7,8	+	+	-	-	-	-	-	+	-	-	-	-	+	-	-
2,5,Te60	-	+	+	+	+	+	+	+	-	+	-	-	+	-	-
11,W32,12,Te50	-	-	-	-	-	+	-	-	+	+	+	+	-	-	-
3,±W29,12,17	-	-	-	-	+	+	+	-	+	+	+	+	-	+	+
1,2,8,12	+	+	+	-	+	+	+	+	+	+	+	+	-	-	-
1,3,8,Te50	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3,±28,7,W15	-	-	+	-	-	-	-	-	+	+	-	-	+	-	+
9,28,17,27	-	-	-	-	-	+	+	-	-	-	-	+	-	-	+

* Under each antiserum, the first column is the CYT reaction with each cell, the second column is the CAT reaction.

TABLE 17

RANGE OF CELLS REACTIVE BY CYT AND CAT*

Number of Panel Cells Marked By CYT

Number of panel cells marked by CAT		0	1	2	3	4	5	Total sera re- active by CYT
	0	295	21	2	3	-	-	26
	1	25	<div>11</div> 12	<div>11</div> 3	-	-	-	9
	2	6	8	<div>3</div> 2	4	2	-	
	3	2	4	5	<div>2</div> 4	-	-	
	4	3	5	6	<div>2</div> 5	1	-	
	5	1	1	2	1	2	2	
Total sera re- active by CAT		37	39					<div>9</div> 21

* A total of 436 multiparous sera were tested by CYT and CAT. 295 were non-reactive, 141 had some positive reactions. The diagonal line indicates sera reacting with the same cells by CYT and CAT (21 sera). Below any box on the diagonal is the number of sera reacting equally with this number of cells but having extra CAT reactions with 1, 2, 3, or 4 cells. Above the diagonal shows the same for CYT. In the small boxes are the number of sera reacting with extra CYT on some cells and CAT on other cells - e.g. The box with "3" in it refers to three sera reacting by CYT and CAT with one cell, with one extra CYT and one extra CAT reaction.

more CAT reactions than CYT) is more common than CYT dominance in the ratio of 2:1 (76:35). In over 5/7 of the sera, there is a predominance of either CYT or CAT reactions. In only 1/7 of reactive sera, CYT and CAT reactivity is equal and on the same cells.

iii. A further analysis was performed on the study of 253 sera from multiparous women as illustrated in Figure 10. The individual cell reaction patterns of each of the 86 positive sera were examined (i.e., one cell reaction pattern representing a serum tested against one cell by the three methods). There were a total of 460 cell reactions, 195 of which were positive by at least one test. The percentages of positive sera or cell reaction patterns falling into each CYT, CAT, AGG classification are shown on Table 18. The classification by serum reactions and by individual cell reactions are not significantly different by chi-square analysis. There is a scarcity of sera and cell reactions which are positive by CYT and AGG but not CAT. It is of interest to note that by testing only by cytotoxicity, as most laboratories do, one would fail to detect 43 positive sera (50% of the positive sera or 17% of all 253 parous sera tested) and 109 positive cell reactions (56% of positive cell reactions or 23.7% of all cell reactions from positive sera) (Table 18).

The 195 positive cell reactions were grouped according to which tests were positive (Figure 11). These cell reaction patterns were then further classified as to which type of serum the cell reaction pattern was from (colours in Figure 11). For example, 27 of the cell reactions were positive by CAT and AGG. Of these, 14 were from sera that were classified as CAT+AGG+ (blue) and the remaining 13 were from sera

TABLE 18
PERCENT OF SERA AND CELL REACTION PATTERNS IN EACH
CYT, CAT, AGG CLASSIFICATION

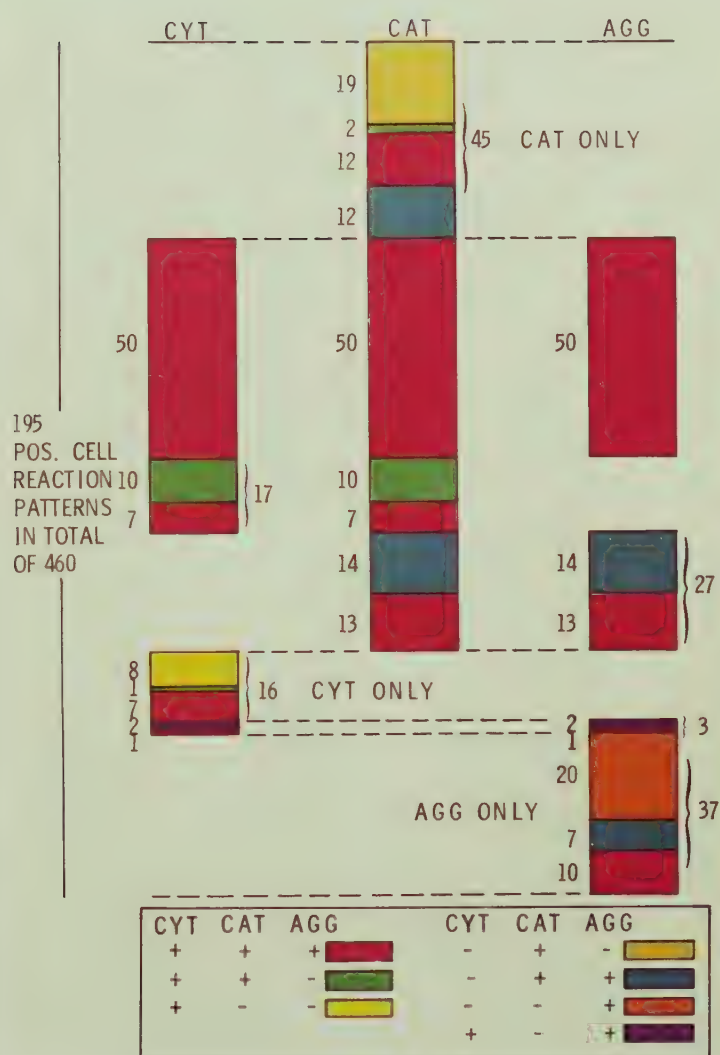
CYT	CAT	AGG	% of 86 positive sera in each classification	% of 195 positive cell reaction patterns in each classification
+	+	+	33.7	25.6
+	+	-	8.1	8.7
+	-	-	5.8	8.2
-	+	-	16.3	23.1
-	+	+	15.1	13.8
-	-	+	18.6	19.0
+	-	+	2.3	1.5

} 50% of
positive
sera

} 55.9% of
positive cell
reactions

FIGURE 11

CYT, CAT, AGG PATTERNS OF 195 POSITIVE
CELL REACTIONS OF 86 ANTISERA.



classified as CYT+CAT+AGG+ (red). About half of each type of cell reaction pattern was in sera exhibiting this pattern also. Similarly, the cell reaction patterns of about half of each classification of sera were of the same type as the serum itself. For example, there were 100 positive cell reactions in the CYT+CAT+AGG+ sera classification (red). Fifty of these were positive by all three methods, another 50 were distributed among the other types of reaction classifications.

Conditional probabilities of positive cell reactions for each test were determined from this data on antisera from parous women (Table 19). When the condition is that only one other test is positive, there are two probabilities which stand out as different from the rest. When CYT was positive, there was a high probability of .779 that CAT would also be positive and .814 that CAT or AGG would be positive. When the condition is that two other tests are positive, there was a very high probability of .943 that CAT would be positive if the other two tests were positive. This re-emphasizes the fact that there is very seldom a CYT and AGG positive reaction without a CAT positive reaction also. Though it might appear from this that the CAT test could be omitted if both CYT and AGG were performed, there are also 23.1% of positive cell reactions which are CAT positive only. Similarly, if only CAT was employed, the 8.7% detected by CYT only and 19% by AGG only would not be picked up (Table 18).

The last group of probabilities show that CAT was positive in .193 of CYT negative cases, AGG was positive in .171 of CYT negative cases, and because there was an overlap of the two, the probability of one or the other being positive was 0.291 when CYT was negative.

iv. Another aspect of this analysis of reactions of 253 parous sera

TABLE 19

CONDITIONAL PROBABILITIES OF POSITIVE CELL REACTIONS
AMONG THE 86 POSITIVE SERA FROM PAROUS WOMEN

<u>CONDITION</u>			<u>PROBABILITY</u>
		CYT+	.187
		CAT+	.302
		AGG+	.254
		CAT+ or AGG+	.389
CYT+	given	CAT+	.482
CYT+		AGG+	.453
CAT+		CYT+	.779
CAT+		AGG+	.658
AGG+		CYT+	.616
AGG+		CAT+	.553
CAT+ and/or AGG+		CYT+	.814
CYT	given	CAT+, AGG+	.649
CAT+		CYT+, AGG+	.943
AGG+		CYT+, CAT+	.746
CAT+	given	CYT-	.193
AGG+		CYT-	.171
CAT+ and/or AGG+		CYT-	.291

was investigated. This is the possibility of the cell influencing the type of CYT, CAT, AGG pattern, rather than the serum being totally responsible. The five major cell donors used in the cell panel were examined for their patterns of reaction with the sera (Table 20). The average percent of sera which were positive by CAT or AGG but not CYT was 8.3%, but the range was from 4.7% to 12.3% among the cell donors. These would be reactions not detected if cytotoxicity alone was performed. Chi-square analysis of cell donor reactions shows a significant difference ($p < 0.01$) between their CYT reactivity as compared to their reactivity by CAT and/or AGG (Table 21). JHAY and LOLS are more highly reactive by CYT, whereas the other three react less frequently by CYT. This difference in reactivity was not seen with sera from patients on dialysis (Table 21).

v. An Eskimo family (Tingmiak) from Inuvik, Northwest Territories, was chosen for another study. The two parents and 11 children are all HL-A identical by cytotoxicity. Most of the family was tissue typed in 1970, along with many other Inuvik Eskimos. The typing was performed on cells reclaimed from liquid nitrogen storage. At that time four of the children were believed to have extra antigens (HL-A1 and 2) and one was shown not to be a biological child of these two parents and was thereafter omitted. Upon repeating the typing in March 1972, all members of the family possessed only an HL-A9 and Te60, when typed with our own typing sera, those of Terasaki (Research Tray 1), and those of Falk, including some Eskimo-specific typing sera of Kissmeyer-Nielsen.

CYT-CAT testing. A panel of 34 sera, screened by both methods, was chosen on the basis of how they reacted by CYT and CAT - most were

TABLE 20

REACTIONS OF SERA FROM PAROUS WOMEN WITH RESPECT TO THE CELL DONOR

		Cell Donor				
		JHAY	LOLS	PMcC	LPOU	MSIH
Sera tested		253	253	253	253	203
Percent positive		(30) 11.9	(37) 14.6	(43) 17.0	(42) 16.6	(24) 11.8
Percent negative		(223) 88.1	(216) 85.4	(210) 83.0	(211) 83.4	(179) 88.2
Percent positive sera in each classification						
CYT	CAT AGG					
+	+	23.3	35.1	18.6	26.2	8.3
+	+	23.3	5.4	2.3	2.4	20.8
+	-	13.3	13.5	4.7	7.1	4.2
-	+	23.3	16.2	30.2	28.6	33.3
-	+	0.0	5.4	18.6	14.3	16.7
-	-	16.7	18.9	23.3	21.4	16.7
+	-	0.0	5.4	2.3	0.0	0.0
Of total sera, percent positives not detected by CYT		4.7	5.9	12.3	10.7	7.9

TABLE 21

CHI-SQUARE ANALYSIS OF REACTIONS BY CELL DONOR

(a) Sera From Parous Women

Reaction Pattern	Cell Donor				
	JHAY	LOLS	PMcC	LPOU	MSIH
CYT positive	18	22	12	15	8
CAT and/or AGG positive, but not CYT	12	15	31	27	16

$$\chi^2 = 13.47$$

$$p < 0.01$$

(b) Sera From Patients on Dialysis

Reaction Pattern		Cell Donor			
	JHAY	LOLS	PMcC	LPOU	MSIH
CYT positive	75	48	42	55	10
CAT and/or AGG positive, but not CYT	45	23	27	18	6

Not significant

reactive with a greater range of cell types by CAT than CYT. All sera reacted identically with all cells by cytotoxicity. Eighteen sera were negative and five sera were positive by both methods, three were positive by CAT only and one by CYT only, and four were CYT negative but showed differences between members of the family when tested by CAT. Three additional sera were first placed in this group, but were later considered to be too variable, from repeat tests performed on blood sent down from Inuvik. The reactions of the group of seven sera positive by CAT only are shown in Figure 12. Three, designated by A, are positive with all members of the family. The other four discriminate between individuals. The father and eight siblings form the largest group, reacting with all the sera; the mother and one son (Abel) form the second group, and sons Raymond and Glen are the third and fourth groups. The seven sera which are cytotoxically negative but CAT positive to at least some members of the family indicate something different from HL-A is being detected. However, the large excess of one group (8/11 siblings) shows the present limitations of capillary agglutinating differentiation, presumably due to insufficient oligospecific capillary agglutinating sera.

Mixed lymphocyte culture (MLC) and skin grafts. MLC cultures were set up as described by Bain et al (1964), and using the Ficoll-Isopaque method of lymphocyte separation (Thorsby, 1969). X-irradiation of one cell type at 8000 r was used to convert the reaction into a unidirectional MLC (UMLC). Cells were in a 1:1 ratio, 0.5×10^6 cells from each donor, in Medium 199 (Microbiological Associates, Bethesda, Maryland), with 15% normal ABO-compatible serum. The cell suspensions were incubated in triplicate in 12 x 75 mm disposable plastic test tubes,

FIGURE 12

DISTRIBUTION OF CAT POSITIVE SERUM REACTIONS
WITHIN THE TINGMIAK FAMILY

RUFUS (A)*					SARAH (AB)				
9,Te60 ^s					9,Te60				
A					A				
B					-				
C					-				
D					-				
E					-				
I				†	2				

Winnifred (B)	Trudy (AB)	Raymond (A)	Nadia (A)	Merlin (A)	Pat (A)	Glen (AB)	Lorna (B)	MaryLou (AB)	Abel (A)	Rhoda (A)
9,Te60	9,Te60	9,Te60	9,Te60	9,Te60	9,Te60	9,Te60	9,Te60	9,Te60	9,Te60	9,Te60
A	A	A	A	A	A	A	A	A	A	A
B	B	B	B	B	B	-	B	B	-	B
C	C	C	C	C	C	C	C	C	-	C
D	D	-	D	D	D	D	D	D	-	D
E	E	E	E	E	E	-	E	E	-	E
I	I	3	I	I	I	4	I	I	2	I

* ABO red blood cell group

^s HL-A antigens

† Numerical classification by CAT reactivity with antisera
A (three sera), B,C,D,E (one serum each).

for five days at 37°C in a 5% CO₂ humidified atmosphere. ³H-thymidine was then added, 1 µc per culture. Incorporation was stopped after three to four hours. The TCA insoluble precipitates were collected and counted in a liquid scintillation counter.

Three groups of UMLC's were performed, one before the grafting and two following the grafting. The final series involved UMLC's duplicating all the skin graft combinations. The four groups of UMLC non-stimulating siblings as obtained from all these experiments are shown on Table 22. Between groups, between any group member + either parent, and between parents, stimulation always occurred. Six of the CAT-identical group of eight are "MLC-identical" also. Raymond, Glen and Abel, who each were in separate CAT groups, are again in separate MLC groups. The number of individuals involved is very small, but there is an indication that CAT and MLC are detecting the same entities.

Based on the CAT results, and keeping within the ABO system, 6 mm punch biopsy skin grafts were performed. Rejection times were judged by eye and photographs, and these are shown on Table 23, classified according to CAT and UMLC groups. There is a significantly lower mean survival time where grafting is within the CAT or UMLC groups - thus there is an inverse correlation of CAT and UMLC with skin graft results. However, the sample size is very small.

b. Correlation with inhibitory antibody

Since there was an indication that the presence of CAT antibody was beneficial to the progress of kidney transplant recipients (Table 8), we examined the possibility of correlation between CAT and the antibody which inhibits unidirectional mixed lymphocyte culture (UMLC) (Ceppellini et al, 1971; Hattler et al, 1971).

TABLE 22
TINGMIAK CHILDREN GROUPED ACCORDING TO UMLC
NON-STIMULATORY GROUPS

GROUP:	I	II	III	IV
	Lorna (1)*	Abel (2)	Raymond (3)	Glen (4)
	Mary Lou (1)	Trudy (1)		Winnifred (1)
	Rhoda (1)			
	Pat (1)			
	Nadia (1)			
	Merlin (1)			

* Numerical classification by CAT reactivity

TABLE 23
SKIN GRAFT SURVIVAL AMONG THE TINGMIAKS, ACCORDING TO CAT AND UMLC GROUPS

	CAT		UMLC	
	Within CAT Groups	Between CAT Groups	Recipient Not Stimulated by Donor	Recipient Stimulated by Donor
	24 23 23 21 19 14	35 35 34 30 30 30 25 23	21 19 14	35 35 34 30 30 30 25 24 23 23 23
Mean Survival in Days	20.7±3.7	30.3±4.4	18.0±3.6	28.4±4.9
+ value	4.37		4.06	
p	<0.01		<0.01	

i. Multiparous sera - Multiparous couples were chosen on the basis of the CYT and CAT reactions of the wife's delivery serum to the cell panel used in the screening. Blood samples were generally obtained at their home, and this six to eight week postpartum serum or plasma and the serum taken at delivery were tested against the husband's cells. Unidirectional mixed lymphocyte cultures were set up as previously described, testing 15% of a 1/10 dilution of the woman's serum. The control was the wife's unirradiated and irradiated cells, in her own serum and normal serum $\left(\frac{AA \times S}{AA \times N}\right)$, to determine percent inhibition of the response to her husband's irradiated cells $\left(\frac{AB \times N - AB \times S}{AB \times N} \times 100\right)$. Though a few sera were heat-inactivated before use, this has been shown not to affect MLC (Stewart et al, 1972). However, heat inactivated plasma may cause non-specific inhibition of MLC (Stewart et al, 1972), and we eliminated the one experiment where inactivated plasma was used (92.8% inhibition).

Table 24 shows the percent inhibition of the sera, grouped according to the humoral reactions with the husbands' cells. The percent inhibition was obtained by taking the ratio of the UMLC response in the wife's serum to that in normal ABO-compatible serum. The highest inhibition was found where CYT and CAT, or CAT alone, were positive. Where CYT and CAT were negative, or CYT alone was positive, there was lower inhibition. Table 25 shows that the presence of CAT was significantly correlated ($p < 0.05$) with the presence of inhibitory antibody; not so with CYT (Table 26). For this analysis, the three sera which were previously CYT+CAT+ but negative when tested, were omitted. Inhibition of greater than 65% was considered to be due to inhibitory antibody, and less than 35% was considered a lack of inhibition, as there was a maximum of 35%

TABLE 24

PERCENT INHIBITION OF UMLC BY MULTIPAROUS SERA

Reaction with Husband's Cells	CYT+ CAT+	CYT- CAT-	CYT+ CAT-	CYT- CAT+
Thibodeau	81.6%	*Grygus	36.4%	28.2%
Zachowski	0	*Fahlman	68.2	19.6
Gallagher	86.7	Stauffer	39.6	
Love	90.0	Staines	19.5	
Drummond	81.1	Page	53.9	
Climenhaga	83.0			
Lockwood	54.2			
Whitby	77.3			
Van Dusen	89.8			
Milligan	73.5			
Average Percent Inhibition \pm S.D.	70.76 \pm 28.6	43.52 \pm 18.4	23.90 \pm 6.08	86.96 \pm 8.47

* Previously CYT+ CAT+

$$\% \text{ Inhibition} = \frac{\text{UMLC response in presence of wife's serum}}{\text{UMLC response in presence of normal serum}} \times 100 \quad \text{or} \quad \frac{\text{ABxN-ABxS}}{\text{ABxN}} \times 100$$

TABLE 25

STATISTICAL CORRELATION BETWEEN CAPILLARY AGGLUTINATION
AND UMLC INHIBITION

Capillary Agglutination	UMLC Inhibition (>65%)	Lack of UMLC Inhibition (<35%)
Positive	10	1
Negative	0	3

$$\chi^2 = 5.6105$$

$$p < 0.05$$

TABLE 26

LACK OF CORRELATION BETWEEN CYTOTOXICITY AND
UMLC INHIBITION

Lymphocytotoxicity	UMLC Inhibition (>65%)	Lack of UMLC Inhibition (<35%)
Positive	8	3
Negative	2	1

Not significant

inhibition of the wife's cells by her own serum. This correlation with CAT, and lack of correlation with CYT, was found whether these limits were >65% and <35%, >70% and <30%, >50% and <50% or >50% and <35%, and regardless of whether the sera previously CYT+CAT+ were omitted.

ii. IgG from dialysis patients - The IgG's of 10 patients on hemodialysis were tested for inhibitory antibody in UMLC. The cultures were set up as previously described, IgG from the patient's serum being tested in a concentration equivalent to that in serum at a 1:10 dilution. The culture medium always contained 15% normal serum. The responder cells (A) were usually surrogate cells of HL-A phenotype close to the patient's, and the stimulating cells (B-1 and 2) were chosen on the basis of humoral reactions (i.e., if a serum was positive, one cell type is chosen to which it is positive and one cell to which it is negative).

There was no pattern of inhibition compared to CYT or CAT results (Table 27). The effect of the IgG on the patient's own cells was determined as a control ($\frac{AA \times S}{AA \times N}$) for inhibition of the response with the stimulating cells ($\frac{AB \times N - AB \times S}{AB \times N} \times 100$). Two patients where there was a stimulation of their own cells were eliminated, and the one case of strong inhibition (94.8%) was in the presence of no humoral antibody.

iii. IgG from transplanted patients - The IgG's of 11 patients with kidney transplants were also tested for inhibitory antibody. The responder cells were usually those of the patient, although in two cases surrogate cells of similar HL-A phenotype were used. There were two different stimulating cells: 1) one sharing one or more HL-A antigens with the kidney donor, and 2) one sharing no antigens with the donor. The IgG of two of the patients stimulated their own cells significantly

TABLE 27

PERCENT INHIBITION OF UMLC BY IgG FROM DIALYSIS PATIENTS

Patient	Stimulating Cell	CYT	CAT	$\frac{AA \times S}{AA \times N}$	ABxN	ABxS	Percent Inhibition*
I	1	+	+		30176	30491	0
	2	+	+	.87	36082	35618	0
II	1	-	-		30176	31304	0
	2	-	-	.71	36082	31350	13.1
III	1	-	-		9783	12667	(+30)
	2	+	-	.93	12084	11620	0
IV	1	-	-		24548	18396	25.1
	2	+	+	1.36	12630	5952	52.9
V	1	-	-		16671	867.5	94.8
	2	+	+	.59	13068	8251.4	36.9
VI	1	-	-		30176	30170	0
	2	-	-	.95	36082	31788	12
VII	1	-	-		9783	10720	0
	2	+	-	1.19	12084	15039	0
VIII	1	-	-		12813	13195	0
	2	-	-	1.25	9854	10846	0

* Percents in brackets are stimulatory rather than inhibitory.

$$\text{Percent Inhibition} = \frac{ABxN - ABxS}{ABxN} \times 100$$

$\left(\frac{AA \times S}{AA \times N}\right)$, and they were thus eliminated. Of the remaining nine, only one had inhibition of above 40% with both stimulating cells, and the IgG in this case was CYT and CAT negative (Table 28).

c. Correlation with antibody dependent cell immunity (ABCIL)

Multiparous couples have also been studied in an antibody dependent cell immunity system, measuring the total immune response due to humoral and cellular components, and these results were compared to those from the cytotoxic and capillary agglutinating tests. The technique is similar to that of Wunderlich et al (1972). "Target" cells, cells of the husband, are labeled with ^{51}Cr . The "killer cells" (wife's cells) and target cells are mixed in a ratio of 40:1 and dispensed into petri dishes. The wife's serum (or plasma) is added in a 1/10 total dilution, with the total serum or plasma concentration being 20%. After an incubation of 4 to 7 hours, chromium release is assayed, in the supernatant fluids after centrifugation. Percent ^{51}Cr release is free ^{51}Cr in the supernatant divided by the total in the supernatant and cell pellet. Maximum ^{51}Cr release, obtained by four freeze-thaw cycles, is 68 to 80% of the total. Spontaneous release varies from 3% to 15% of the total. ABCIL is evidenced by an increase in ^{51}Cr release, after this is adjusted to account for spontaneous release.

Eighteen sera were tested (Table 29). All sera with capillary agglutinating (CAT) and cytotoxic (CYT) antibodies had ABCIL activity. With no antibodies, there was usually no ABCIL. In one case, ABCIL was detected after antibodies had disappeared, and in another, ABCIL was present where CAT, but not CYT, antibodies were present. In a further case, where CYT antibodies only were present, ABCIL was negative. A

TABLE 28

PERCENT INHIBITION OF UMLC BY IgG FROM TRANSPLANTED PATIENTS

Patient	Stimulating Cell	CYT	CAT	$\frac{AAxS}{AAxN}$	ABxN	ABxS	Percent Inhibition*
I	1¶	-	-	.97	364	299	8
	2§	-	-		7861	7623	0
II	1	-	-	.896	37877	37325	0
	2	±	±		43189	43433	0
III	1	-	-	1.08	2611	2807	0
	2	-	-		2361	1887	20
IV	1	-	-	.83	5728	7690	(+34)
	2	-	-		6030	6444	0
V	1	ND	ND	1.06	3471	4374	(+26)
	2	+	±		2896	2868	0
VI	1	-	-	.89	2910	1643	43.5
	2	-	-		2254	1352	40
VII	1	-	-	1.13	5259.3	5836.1	(11)
	2	-	-		4509.5	5090.6	(13)
VIII	1	-	-	1.32	6359.8	7306.6	(12)
	2	-	-		6555.8	7461.6	(14)
IX	1	-	-	1.10	2015.8	3996.6	(98)
	2	-	-		4358.0	5858.1	(34)

* Percents in brackets are stimulatory rather than inhibitory.

¶ Stimulating Cell 1 shares antigens with kidney donor.

§ Stimulating Cell 2 shares no antigens with kidney donor.

$$\% \text{ Inhibition} = \frac{ABxN - ABxS}{ABxN} \times 100$$

TABLE 29

CHROMIUM RELEASE FROM HUSBAND'S CELLS IN THE
PRESENCE OF MULTIPAROUS SERA

Reactions with Husband's Cells	CYT+ CAT+	CYT- CAT-	CYT+ CAT-	CYT- CAT+
Adjusted percent ⁵¹ Cr release for each husband-wife combination	14.0	0.7	7.3	16.8
	22.4	3.7		
	23.4	-1.1		
	23.6	-1.8		
	24.0	*16.0		
	24.9			
	27.6			
	32.2			
	39.5			
	44.5			
	54.0			

* Serum previously CAT+ CYT+

chi-square analysis to determine the correlation of CYT or CAT with ABCIL, taking greater than 15% Cr release as evidence of ABCIL activity, showed that the presence of both CYT and CAT are significantly associated with ABCIL activity. Thus, neither CYT nor CAT could be implicated in the ABCIL reaction. However, there were very few sera tested which were positive by one method only.

4. Class of Immunoglobulin

a. Introduction

IgG and IgM were prepared from four sera which had CYT and CAT antibodies. From an additional sixteen sera, IgG only was prepared. IgM separation was performed on Sephadex G-200 (Weir, 1967). IgG was prepared on Sephadex G-200 in very few instances, and on DEAE - Sephadex for the majority of sera (Baumstark, 1964). IgG was frozen in aliquots at -20°C and thawed only once before testing. IgM was usually tested immediately after preparation. The preparations were generally adjusted to the normal serum concentration of the immunoglobulins: 8 to 17 mg/ml for IgG and 0.5 to 1.9 mg/ml for IgM (Cohen and Milstein, 1967). Immuno-electrophoresis was used to confirm the presence and purity of immunoglobulin in the preparations. The most common impurity was transferrin.

b. Results (Table 30)

In a total of six tests of each fraction with a cell type against which antibodies were present in the serum, all six showed antibody in the IgG preparation, to a titre comparable to that of the serum. Two of these cases also showed some activity in the IgM fraction, but these were to a much lower titre than either the serum or IgG. This can be explained by IgG contamination of the IgM preparations, but we did not test for

TABLE 30

CAT AND CYT REACTIONS TO EIGHT SERA AND THE IgG AND IgM FROM THESE SERA

Patient		Titre of Antibody Detected	
		CAT	CYT
I	serum 7.7 mg/ml IgG 8.2 mg/ml IgM	1/2 1/4 -	1/2 conc -
II (a)	serum 11 mg/ml IgG 1.4 mg/ml IgM	1/2 conc -	conc conc -
(b)	serum IgG IgM	1/8 1/8 conc	1/16 1/16 1/4
(c)	serum IgG IgM	1/2 1/4 -	1/2 1/4 -
III	serum 14 mg/ml IgG 1.3 mg/ml IgM	1/4096 1/8192 1/32	1/8192 1/8192 1/128
IV	serum 14 mg/ml IgG 1.5 mg/ml IgM	conc conc -	conc 1/2 -
V (a)	serum 12.6 mg/ml IgG	+	+
(b)	serum 19.4 mg/ml IgG	conc - -	1/4 + -
VI	serum 18.3 mg/ml IgG	+	+
VII	serum 8.1 mg/ml IgG	1/4 -	conc 1/4
VIII	serum 6.6 mg/ml IgG	- -	+ conc
12 patients	serum all ~14 mg/ml IgG	- -	- -

this possibility. In two further tests of IgG with cells reactive to the sera by CYT and CAT, there was both CYT and CAT activity in the IgG. In three cases where the serum was CYT reactive only, only one of the IgG's failed to show cytotoxic activity. In 12 cases where the serum had no antibody detectable, the IgG also showed a negative reaction.

CHAPTER V

DISCUSSION

A. Antigenic Specificity of CAT and AGG

The major conclusion drawn from this thesis is that capillary agglutination (CAT) and leukoagglutination (AGG) are each measuring a different spectrum of histocompatibility antigens than is lymphocytotoxicity (CYT). The major analysis leading to this conclusion is that of the reactions of parous sera. This is based on an entirely random selection of sera, with all sera obtained from the Obstetrical Service being screened. Other published comparisons between CYT and CAT or AGG are based on analyses of sera chosen for their cytotoxic mono- or oligospecificity, lending an instant bias.

This analysis of parous sera, however, is not strictly comparable to that of renal patients on hemodialysis. The latter are usually sensitized by the many units of blood received, thus they have been exposed to a wider range of antigenic specificities and a larger percentage have developed antibody of all three types - CYT, CAT, and AGG.

There are several possible explanations for the discrepant results obtained by the three methods:

1) Are the two agglutination tests subject to many false positives? Table 2 gives technical reproducibility of all three methods - neither agglutination method gives an excess of false positives.

2) Are the two agglutination tests marking non-lymphocyte antigens? This is unlikely, for the following reasons:

a) They are definitely not marking platelet-specific antigens, as there are no platelets in the cell preparation for either method; in fact

in the presence of excess platelets, there is non-specific clumping of the leukocytes.

b) Though there are red blood cell antigens A, B, Rh (Zmijewski, 1968) and I-i (Shumak et al, 1971) on lymphocytes, they are not responsible here. Severson and Thompson (1968) found mouse sera with both leukoagglutinating and hemagglutinating properties present in different titres, and also that in the presence of antisera against red blood cells, lymphocytes still migrate though red cells in the suspension do not (Thompson et al, 1968). They also showed, and our results agree, that capillary agglutinating crossmatch results were independent from those predicted by possible anti- A, B or Rh reactions (Thompson et al, 1968). An analysis in our laboratory of 14 sera with leukoagglutinating, capillary agglutinating and cytotoxic activity (of these, 3 sera were not cytotoxic) showed they possessed no antibody against red blood cells.

c) There are granulocytes in the cell preparations, but there is little evidence that the granulocyte-specific antigens NAI, NBI and NCI (Lalezari et al, 1970) are being marked in capillary agglutination. Thompson et al (1970) state that either pure lymphocytes or a leukocyte mixture can be used in capillary agglutination. In a later report, they show that in the case of one capillary agglutinating serum there was no relation to NAI, NBI or NCI (Thompson et al, 1971). Upon further analysis, they found this serum to contain an antibody to NBI and another of unknown specificity (Thompson, personal communication).

In our laboratory, 14 sera known to have capillary agglutinating activity were tested against a pure lymphocyte preparation. In no instance was there a negative reaction (Chapter III). Leukoagglutination

however, does detect these granulocyte-specific antigens (Lalezari and Bernard, 1966) and the technique requires the presence of some granulocytes (Zmijewski, 1968).

3) Are the two agglutination tests marking HL-A antigens but with increased sensitivity? It does not appear that either of these methods is consistently more sensitive than cytotoxicity (Tables 11-12), but rather that each method is more sensitive in detecting antibody in some antisera. Further, Figures 8 to 11 show that there is not a simple "inclusion" of antibodies detected by one method over another, but rather an overlap of each method by the other two, each detecting antibody in some sera where the others do not. This is in contrast to the results of Thompson et al (1970), who found CAT positive whenever CYT or AGG was. However, this was only with 10 sera, chosen for their cytotoxic monospecificity.

4) If different antigens on the lymphocytes are being detected by the agglutinations compared to cytotoxicity, what are they?

a) There are some HL-A antigens (6a, 7c) [Fig. 1, Appendix A] and non-HL-A antigens (5a, 5b) identifiable by leukoagglutination but not cytotoxicity. It is possible that capillary agglutination is affected by these, however the antigens were identified by leukoagglutination and thus the sera with capillary agglutinating activity only cannot be explained. Thompson provided evidence that 5a and 5b antigens are not related to the results of one CAT antisera (Thompson et al, 1971), but he does find that anti-5a and 5b sera are reactive in capillary agglutination (Thompson, personal communication).

b) Van Rood's 4a and 4b antigens are also linked with the other HL-A antigens (Fig. 1, Appendix A). Since these just involve second

locus antigens, it is unlikely that the discrepancies could all be explained on the basis of 4a and 4b antigens. In the analysis of the Tingmiak family, capillary agglutinating sera showed differences between siblings with HL-A9 and Te60. Since the second sublocus antigen Te60 belongs to the 4b supertype, the 4a and 4b system cannot be implicated in that particular analysis.

c) Most of the HL-A antigens have known cross-reactivity with other antigens of the HL-A system (Fig. 2, Appendix A). There is the possibility that capillary agglutination and/or leukoagglutination may detect these cross-reactive antigens in some cases. This has not yet been analysed.

d) An MLC locus has recently been postulated (Dupont et al, 1971; Yunis et al, 1971), its antigens being associated with HL-A but governing the MLC response. There is some evidence that capillary agglutination also marks these antigens (Table 22).

B. Enhancing Antibody

Of the various types of antibody formed against histocompatibility antigens, they are not all deleterious. There has been an "enhancing" antibody described by Voisin et al (1969), and this may be responsible for inhibition of UMLC (Miller et al, 1971; Gordon et al, 1971). This antibody is non-cytotoxic, non-complement-fixing, and is an IgG molecule (Voisin et al, 1969). The "blocking antibody" of Hellström may be an antigen-antibody complex (Sjögren et al, 1971). Capillary agglutination could also be caused by these complexes. The extent to which lymphocytotoxicity, leukoagglutination and capillary agglutination are implicated as "enhancing antibody" is also discussed here:

1) The two agglutinating methods are complement independent, with sera inactivated before use and no complement added to the system. In cytotoxicity however, sera are not inactivated, and rabbit complement is used to effect death of cells where specific antibody is present.

2) Cytotoxicity and capillary agglutination both have IgG activity rather than IgM (Table 30). Leukoagglutination was not tested, but was found by other investigators to possess both IgG and IgM activity (Table 1).

3) Capillary agglutinating (CAT) antibodies were shown to correlate with a beneficial effect on the progress of kidney transplant recipients (Table 8). In addition, the presence of CAT antibodies in the sera of multiparous women showed significant correlation with antibody inhibitory in UMLC (Table 25). Cytotoxic antibody on the other hand, showed a lack of correlation with both of the above (Table 9 and 27), and, in fact, preformed CYT antibody has been shown to cause hyperacute rejection in kidney transplant recipients (Kissmeyer-Nielsen et al, 1966). It should be noted that there have been only two cases reported of a kidney allograft where CAT was positive, and in both there was subacute but progressive rejection (Thompson et al, 1970). Leukoagglutination was not included in the analyses of UMLC inhibitory antibody or influence on rejection.

4) Capillary agglutination, then, parallels the definition of "enhancing" antibody, as it is a non-cytotoxic (since the CYT and CAT specificities are different), non-complement-fixing IgG which correlates with inhibition of UMLC, and has a beneficial effect on transplant recipients. Leukoagglutination also has many of the same properties, but the

latter two studies were not performed with this test.

C. Speculation

The biological significance of the capillary agglutinating (CAT) and leukoagglutinating (AGG) antibody needs further study. The 4a, 4b, 5a, 5b, 6a, and 7c sera of van Rood and NAI, NBI, NCI sera of Lalezari should be thoroughly investigated using CAT and AGG, to determine whether these might explain the differences in reaction. The possibility of cross-reacting HL-A antigens playing a role must be examined more closely, as must the role of the cell donor. CAT, CYT and AGG must also be conclusively analysed as to their association with the "UMLC locus", with the antibody inhibitory to UMLC, with antibody dependent cell mediated immunity (ABCIL), and with Hellström's colony inhibition assay (G.E. Pierce et al, 1971).

To determine whether antigens detected by CAT or AGG segregate with the HL-A antigens, we are planning to perform family studies, with the leukocytes of each member tested with a panel of sera by CYT, CAT and AGG. Groups of HL-A identical siblings are also being tested in the same way, to detect possible non-HL-A antigens. Throughout these and other studies, the interest, of course, is on the donor-recipient relationship in transplantation. However, all information cannot be gleaned by studying this relationship. Besides the family members used in the above study, the multiparous woman and her husband are extremely useful for most other analyses. Using these couples is much simpler than using renal patients who have received many transfusions, or transplant recipients who are on immunosuppression, since with multipara only four HL-A antigens foreign to the donor are involved.

CHAPTER VI

SUMMARY AND CONCLUSIONS

The value of leukocyte capillary agglutination in transplant immunology was studied, by comparing it with lymphocytotoxicity and leukoagglutination in various systems. In all of the test situations, it became evident that lymphocytotoxicity, capillary agglutination and leukoagglutination are detecting different types of antibodies.

A. Anamnestic Response

The humoral response of postmenopausal women to blood transfusions led to important observations concerning the "anamnestic" response to HL-A antigens.

1. Sensitization to HL-A antigens to produce a humoral response may become latent in the course of time, but immunologic memory persists as shown by the humoral response to subsequent exposure. This is shown in the case of the postmenopausal multiparous woman who receives a blood transfusion.
2. Antibodies detectable by capillary agglutination (CAT), lymphocytotoxicity (CYT), and leukoagglutination (AGG) are formed, but none of the methods detect antibody in the latent state. However, in at least two of four cases, CAT and AGG antibodies are detectable after a longer period of time than are CYT antibodies.

B. Characterization of CAT Antibodies

CAT antibodies are induced by allogeneic histocompatibility antigens (other than red blood cell antigens or granulocyte antigens),

which are to a certain, but presently indefinable, extent independent of HL-A. The technique detects a different antigenic spectrum than cytotoxicity or leukoagglutination, but the biological significance of this requires further study. Whether CAT antibodies are finally recognized as either harmful or beneficial, they certainly have an important role in transplantation, and thus the technique cannot be neglected. Technically, capillary agglutination, lymphocytotoxicity and leukoagglutination are of comparable reproducibility, except that leukoagglutination is less reproducible when cells are tested at intervals of time. The differences between the techniques are not based on sensitivity differences. The relation between the sensitivity of all three methods changes from serum to serum, with each detecting antibody at a higher titre in at least some sera. There is also no consistent relation between the methods with respect to relative serum changes with the passage of time. CAT antibodies often remain longer, whereas AGG antibodies do not usually remain as long as the other two types. The capillary agglutinating antibody is an IgG molecule, as confirmed by Thompson (personal communication). Cytotoxic activity also resides in the IgG fraction of serum. Where IgM was active in either capillary agglutination or cytotoxicity, it was rapidly diluted out - thus the activity was most likely due to contaminating IgG.

Capillary agglutination and leukoagglutination do not measure the same spectrum of antigenic specificities as lymphocytotoxicity. The three techniques correlate in about one-third of antisera from parous women. In the rest of the sera, antibody is detected by only one or two of the methods. Cytotoxicity has been adopted as the most reliable

technique for antibody detection, but these others may be just as useful, if not more so. However, there is the problem of comparing and correlating them after the HL-A system has been defined in terms of computer analysis of cytotoxic reactions. 50% of the antibodies in parous sera were detected by capillary agglutination (CAT) or leukoagglutination (AGG), but not cytotoxicity (CYT). In positive sera, there is a .291 probability that in a cell reaction capillary agglutination and/or leukoagglutination will detect an antibody when cytotoxicity does not. There is a significant difference between the reactions of the cell donors with parous sera, but not with sera from patients on dialysis. The nature of the cell, then, may in part determine whether a CYT, CAT or AGG reaction will take place.

The Tingmiak family study shows that CAT can differentiate between cytotoxically defined HL-A identical siblings in a manner which shows some correlation with UMLC reactivity. However, identity by one or both of these criteria did not favour graft prolongation.

The studies on inhibitory or enhancing antibody may be summarized as follows:

1. There is an indication that CAT antibodies have a beneficial effect on the progress of kidney transplant recipients. In this study, however, no direct crossmatches were performed using capillary agglutination.
2. Inhibitory antibody in the postpartum sera of multipara was significantly correlated with the presence of CAT antibody. CYT antibody showed no correlation.
3. Inhibition caused by IgG from patients on dialysis or with a renal transplant could not be correlated with the presence

of either CYT or CAT. However, most of these were negative humorally and most showed no inhibition.

Finally, antibody dependent cell mediated immunity against lymphocytes (ABCIL) has been demonstrated in the sera of multiparous women where there are CYT and CAT antibodies. With no antibodies, there was usually no ABCIL. Too few cases were studied where there was only one of the two antibodies detectable, and no correlation was seen with either method.

CHAPTER VII

PRACTICAL APPLICATIONS

A. Anamnestic Response

Since latent immunity to HL-A antigens has been shown in this thesis, there are several implications in the field of transplantation.

1. Humoral sensitization of a given individual is best detected in serum obtained shortly after maximal HL-A antigen exposure and it is this serum as well as the most recent that should be used for the direct recipient serum/donor lymphocyte cytotoxic crossmatch test. This is relevant to the recent decrease in the use of whole blood in hemodialysis units, circumstances under which the serum may lose its cytotoxicity. Caseley et al (1971) have also made this recommendation based on loss of antibody and change of antibody spectrum with time.
2. A non-transfused multiparous woman, without cytotoxic antibodies, probably should not be given an allograft sharing possible sensitizing antigens of her husband.
3. There is however the possibility that these antibodies developing in pregnancy, or associated non-complement dependent agglutinating antibodies, may have an "enhancing" effect on transplants (Beleil et al, in press). It is important to detect non-cytotoxic antibodies for this possible reason, in anticipation that the full significance will be worked out in the not-too-distant future.
4. Memory cells may also remain as a result of a previous renal allograft. This has not been proved by our experiments, since these patients are heavily immunosuppressed and thus not strictly comparable

to the situation of multiple pregnancy or blood transfusions. However, we agree with J. C. Pierce et al (1971) that a recipient should not be given a graft sharing major HL-A mismatches with a previously rejected kidney.

B. CAT as a Tissue Typing Technique

Capillary agglutination is a difficult and exacting laboratory test, and very sensitive to the test conditions. The solutions must be made up monthly as some of them precipitate out with time. In this event, the cells may clump or fail to migrate smoothly. The cell preparations must be very fresh, as after a few hours they give non-specific clumping (confirmed by Thompson, personal communication). Any appreciable delay in preparation after the blood is obtained will cause the same problems (Severson and Thompson, 1968). The sera also must be specially handled to make sure that there is no precipitate to cause non-specific clumping.

As we now use the technique in this laboratory, it is too tedious and takes too long to be used as a routine tissue typing technique. With automation (Thompson does have a special tube holder to load, spin and angle-hold the tubes), the method would be much more easily adapted to routine. Also, a cell preparation which allows extended use of the cells would increase its value. Automatic measurement by a light sensitive device (Thompson et al, 1968) would increase the efficiency of the test.

C. Significance of the CYT, CAT, AGG Discrepancies

Since the three methods, capillary agglutination, cytotoxicity and leukoagglutination, are not detecting the same antigens, there must be a re-evaluation of the standard tissue typing techniques. Leukoagglutination has been eliminated by most investigators in favour of

cytotoxicity, believed to be the most reliable technique for antibody detection and antigen identification. However, both AGG and CAT could prove useful for, in antisera from parous women, there is a probability of .19 that CAT will detect antibody in a cell reaction where CYT does not and .17 that AGG will detect antibody in that situation.

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APPENDIX A

ADDITIONAL INFORMATION ON KIDNEY TRANSPLANT SUCCESS, THE HL-A
SYSTEM, NON-HL-A ANTIGENS AND IMMUNOGLOBULIN CLASSES.

APPENDIX A

Knowledge concerning the HL-A system is constantly being expanded. Table 2 shows the antigens defined on the two HL-A subloci, to date. Figure 2 includes the latest data on cross-reactivity from Dr. P.I. Terasaki's laboratory (September, 1972). The number of antigens known to be involved in cross-reactions is ever-increasing. Figure 1, based on supertypes within the HL-A system, is also a recent development, from the laboratory of Dr. J.J. van Rood (June, 1972).

Terminology is also changing constantly. At the Fourth International Congress of the Transplantation Society (September, 1972), the term "Major Histocompatibility Complex" was coined. In the mouse the two H-2 regions are at opposite ends of the complex, the H-2K and H-2D regions, with the Immune response (Ir) locus controlling MLC reactions being between 2K and 2D, but nearer to 2K. In man, the HL-A subloci are the first (or LA) and second (or Four) subloci; and the evidence favours the MLC locus being outside the two HL-A regions but closely related to the second or Four sublocus. This MLC locus may or may not be equivalent to a skin graft locus. In Dr. F.H. Bach's terms, the serologically defined (SD) antigens belong to the HL-A subloci, and the lymphocyte defined (LD) antigens belong to the MLC locus.

TABLE I
KIDNEY TRANSPLANT FUNCTION OF FIRST ALLOGRAFTS BY
DONOR SOURCE AND YEAR OF TRANSPLANT

Donor Type	Year of Transplant	Sample Size	Percent of Functional Transplants		
			One Year	Two Years	Three Years
Sibling	1951-1966	240	64.4	57.6	53.3
	1967	138	78.9	71.4	66.0
	1968	185	81.0	75.5	71.5
	1969	195	76.7	71.6	69.9
	1970	207	82.5	79.6	-
	1971	109	73.4	-	-
Parent	1951-1966	403	56.7	50.4	45.6
	1967	145	70.9	63.1	57.4
	1968	190	72.9	68.0	60.3
	1969	199	71.7	64.1	58.8
	1970	199	71.4	67.2	-
	1971	102	66.4	-	-
Cadaver	1951-1966	663	35.5	27.9	22.3
	1967	365	44.7	37.7	32.9
	1968	584	46.6	38.9	32.9
	1969	737	54.6	46.0	40.3
	1970	858	54.1	45.7	-
	1971	466	46.7	-	-

Source: Advisory Committee to the Renal Transplant Registry. Tenth Report of the Human Renal Transplant Registry. (In press. Table 2.)

TABLE 2

ANTIGENS OF THE TWO HL-A SUBLOCUS

First Sublocus, or LA

Second Sublocus or "4"

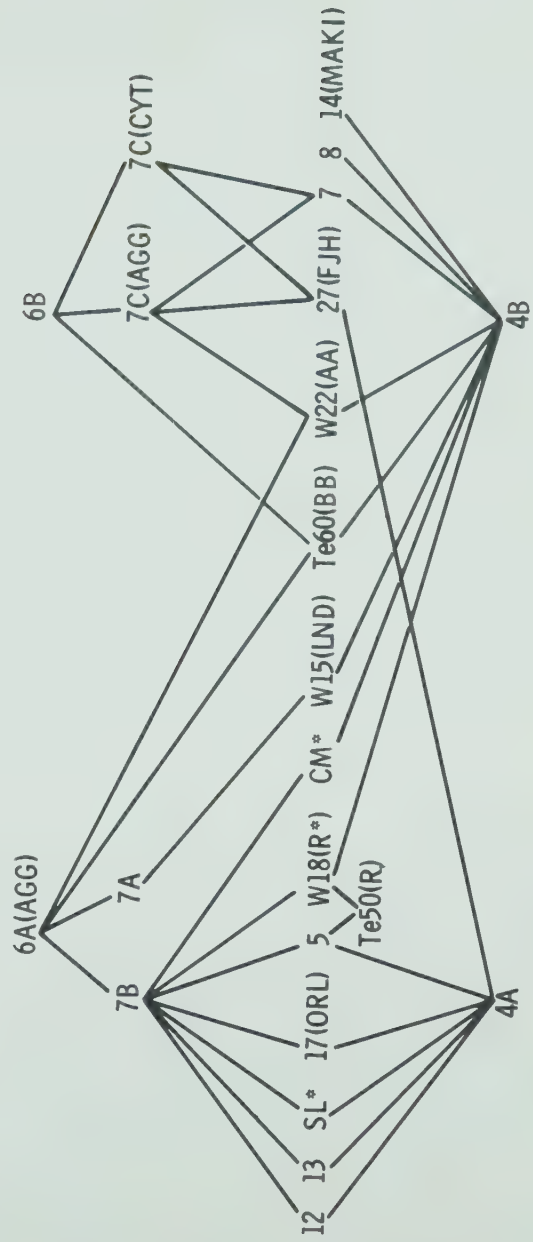
Previous Designations

Previous Designations

HL-A or Workshop Nomenclature	Cepellini				Payne				Cepellini						
	Terasaki	Kissmeyer-Nielsen	Dausset	Payne		Terasaki	Kissmeyer-Nielsen	Dausset	Payne		Terasaki	Kissmeyer-Nielsen	Dausset	Payne	Cepellini
HL-A1	Te1	LA1	Da11	LA1	To8	HL-A5	Te6	4C	Da5	4c	To5				
HL-A2	Te2	LA2	MAC	LA2	To9	HL-A7	Te11	7	Da10	4d	To20				
HL-A3	Te8	LA3	Da12	LA3	To10	HL-A8	-	8	Da8	7d	To7				
HL-A9	Te4	9	Da16	LA4	To12	HL-A12	Te9	KN12	Da4	-	To11				
HL-A10	Te12	KH	Da17	-	To13	HL-A13	Te26	HN	-	-	To21				
HL-A11	Te13	ILN*	Da21	-	To26	HL-A14	Te54	MaKi	Da18	-	To27				
HL-A28	Te40	Ba*	Da15	-	-	HL-A17	Te57	SL	-	-	-				
W29	Te63	-	Da22	-	-	HL-A27	Te52	FJH	-	-	To29				
W31	Te66	-	Da25.2	-	-	W15	Te55	LND	Da23	-	-				
W32	Te59	-	-	-	-	W16	Te64	-	Da31	-	-				
W19.3	-	-	Da25.1	-	-	W18	Te58	4C* (R*)	-	-	-				
W19.5	W48	-	-	-	To30	W21	Te61	ET*	Da24	-	-				
						W22	Te51	AA	-	-	To28				
						Te50	Te5	4C(R)	Da20	-	To25				
						Te60	Te60	BB	-	-	To23				

Source: Fifth International Histocompatibility Conference, Evian, France. June 1972.

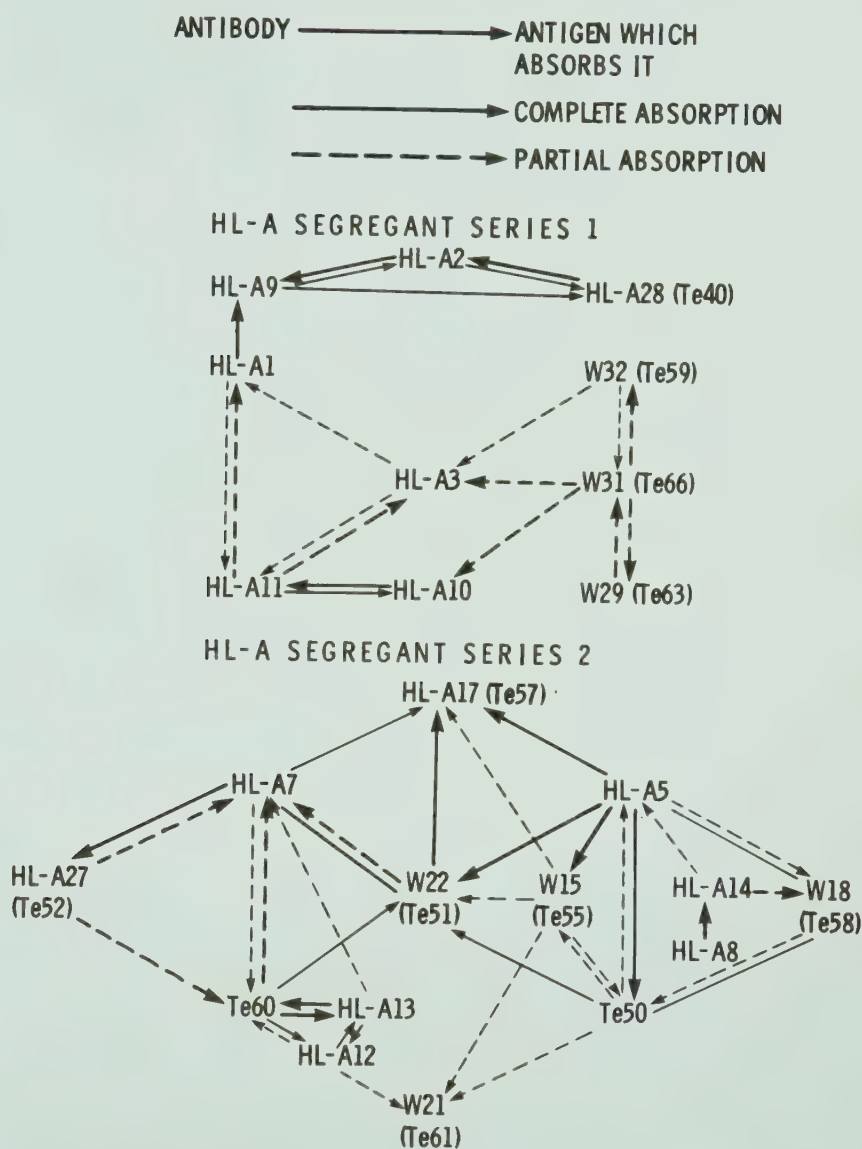
FIGURE 1
SUPERTYPIC SPECIFICITIES WITHIN THE HL-A SYSTEM.



Source: D'Amaro, Keuning, van Leeuwen and van Rood.
Histocompatibility Testing 1972. In press.

FIGURE 2

CROSS-REACTIONS WITHIN THE TWO HL-A SUBLOCUS OR SEGREGANT SERIES.



Source: Sengar, D.P.S., personal communication.
 (Based on data from laboratory of Dr.
 P.I. Terasaki, September, 1972.)

TABLE 3
PLATELET, NEUTROPHIL, AND LYMPHOCYTE SPECIFIC ANTIGENS

Investigators	Antigen	Date Identified	Specificity	Frequency of Occurrence
Van Loghem, Shulman	Zw ^a (PI ^{Al})	1959	platelet	98%
Van der Weerd	Zw ^b	1963	platelet	26%
Van der Weerd	Ko ^a	1961	platelet	15%
Van der Weerd	Ko ^b	1961	platelet	99%
Shulman	PI ^{E1}	1962	platelet	99%
Shulman	PI ^{E2}	1962	platelet	5%
Shulman	PIGrLy ^{BI}	1962	platelet, granulocyte, lymphocyte	46%
Shulman	PIGrLy ^{CI}	1962	platelet, granulocyte, lymphocyte	30%
Shulman	PIGrLy ^{FI}	1965	platelet, granulocyte, lymphocyte	65%
Lalezari	NAI	1965	neutrophil	56%
Lalezari	NBI	1966	neutrophil	99%
Lalezari	NCI	1970	neutrophil	72%
Shulman	Ly ^{DI}	1964	lymphocyte	36%

TABLE 4
CURRENT KNOWLEDGE OF THE THREE MAIN IMMUNOGLOBULIN CLASSES WITH ANTIBODY ACTIVITY

Characteristics	IgG (γ G)	IgM (γ M)	IgA (γ A)
Molecular Weight	160,000	900,000	390,000
Percent of Total Antibody in Serum	80%	5-10%	10%
Sedimentation Constant	7s	19s	11s
Structural Units	1-7s units	5-7s units	2-7s units
Heavy Chain	$\gamma_1, 2, 3$ or 4	μ 1 or 2	α_1 or 2
Light Chain	60% λ , 40% κ		
Properties	-fixes complement -crosses placenta -affinity for cells and tissues	-fixes complement	-resistant to proteolytic enzymes -selective seromucous secretion.
Antibody Reaction Sites	2	5	2

Sources: 1. Cohen, S. and Milstein, C.: Structure and biological properties of immunoglobulins. Adv. Immunol. 7: 1-89, 1967. Table 2, p. 10.
 2. Weiser, R.S., Myrvik, Q.N. and Pearsall, H.N.: Fundamentals of Immunology. Great Britain: Henry Kimpton, 1969. Chapter 5, p. 26.

APPENDIX B

CYTOTOXIC AND AGGLUTINATING ANTIBODIES IN PAROUS WOMEN:
OUR DATA COMPARED TO THAT OF OTHER INVESTIGATORS.

TABLE 5
OCCURRENCE OF CYTOTOXIC ANTIBODIES IN PAROUS WOMEN

Gravida	UCLA Medical Centre Los Angeles, California Terasaki, 1970 ^a	U of A Hospital Edmonton, Alberta April 1970 - August 1972
0	0% - 30 males 20 nulliparous women	0% - 38 males 20 nulliparous women
1	4/24 = 16.7%	33/251 = 13.1%
2	25/106 = 23.6%	32/155 = 20.6%
3	32/89 = 36.0%	65/269 = 24.2%
4	49/110 = 44.5%	44/139 = 31.7%
5	41/95 = 43.2%	19/71 = 26.8%
6	29/46 = 63.0%	8/30 = 26.7%
7+	51/104 = 48.6%	11/43 = 25.5%
	47.8%	29.0%

^aSource: Terasaki, P.I., Mickey, M.R., et al.: Maternal-fetal incompatibility. I. Incidence of HL-A antibodies and possible association with congenital anomalies. Transplantation 9: 538-543, 1970.

TABLE 6
OCCURRENCE OF LEUKOAGGLUTINATING ANTIBODIES IN PAROUS WOMEN

Duke University Medical Centre Durham, North Carolina Zmijewski, 1967 ^a			University of Alberta Hospital Edmonton, Alberta April 1970 to August 1972		
Gravida	Agglutination	Gravida	Agglutination	Cytotoxicity	
1	17/501 = 15.4%	1	38/205 = 18.5%	28/205 = 13.7%	
2	73/296 = 24.7%	2	21/118 = 17.8%	20/118 = 16.9%	
3	57/212 = 26.9%	3	35/169 = 20.7%	34/169 = 20.1%	
4+	122/368 = 33.2%	4	29/93 = 30.1%	25/93 = 26.9%	25.4%
		5	11/49 = 22.4%	13/49 = 26.5%	
		6	5/25 = 20.0%	5/25 = 20.0%	
		7	10/30 = 30.3%	7/30 = 23.3%	
Total	269/1377 = 19.5%		148/689 = 21.5%	132/689 = 19.2%	

^aSource: Zmijewski, C.M., Zmijewski, H.E., et al.: The relationship of the frequencies of white cell antibodies in the sera of multiparous women. Int. Arch. Allergy 32: 574-582, 1967.

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